STIC-ILL

Vol no 613

From: Choi, Frank
Sent: Monday, June 02, 2003 8:21 PM
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-----Original Message-----
From: Choi, Frank
Sent: Monday, June 02, 2003 7:53 PM
To: STIC-ILL
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Importance: High

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Relationship of Serum Copper and Zinc Levels to HIV-1 Seropositivity and Progression to AIDS

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Summary: Dietary, serum, and tissue levels of copper and zinc were determined at baseline in a cohort of homosexual men to investigate the relationship of these factors to human immunodeficiency virus type 1 (HIV-1) seropositivity and subsequent progression to AIDS. Using a nested case control design, 54 asymptomatic HIV-1 seropositives who later progressed to AIDS were compared with 54 HIV-1 seropositives who did not progress and 54 seronegatives (mean follow-up time 2.5 years). Serum levels of copper and zinc were estimated from frozen serum samples, tissue levels from stored toenail samples, and dietary intakes from a semiquantitative food frequency questionnaire administered at baseline. Neither dietary copper and zinc nor their levels in toenails were associated with HIV-1 seropositivity or progression to AIDS. However, serum copper levels were higher ($p = 0.002$) in HIV-1-seropositive progressors (mean = 115.6 μg/dl; SD = 17.1) than the seropositive nonprogressors (mean = 109.0 μg/dl; SD = 15.8) and the seronegatives (mean = 101.9 μg/dl; SD = 16.7). Conversely, serum zinc levels were lower ($p = 0.016$) in the seropositive progressors (mean = 85.2 μg/dl; SD = 11.5) than the seropositive nonprogressors (mean = 90.7 μg/dl; SD = 12.0) and the seronegatives (mean = 92.0 μg/dl; SD = 14.7). Furthermore, in a logistic regression, higher serum copper (odds ratio per 20-μg/dl increase = 2.23; 95% confidence interval = 1.02–4.87) and lower serum zinc (odds ratio per 20-μg/dl increase = 0.30; 95% confidence interval = 0.14–0.66) predicted progression to AIDS independently of baseline $CD4^+$ lymphocyte level, age, and calorie-adjusted dietary intakes of both nutrients. These data suggest that copper and zinc (both of which are acute phase reactants) may be useful markers of progression to AIDS and HIV-1 viral activity. Key Words: Copper—Zinc—Nutritional status—Human immunodeficiency virus seropositivity.

Patients with AIDS are known to suffer from specific nutrient deficiencies, malnutrition, and intestinal malabsorption (1,2), but nutritional status earlier in the course of infection has not been widely studied. There are two key questions to answer. First, can any nutritional deficiencies be identified that might be amenable to intervention, and second, can specific nutritional markers be identified to predict progression to AIDS? During infection, serum zinc levels generally decrease, primarily owing to increased hepatic uptake of zinc (3–5), while copper levels increase owing to increased production of ceruloplasmin (6–8). Both nutrients are believed

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be important in maintaining immune function (9,10). In particular, zinc deficiency has been associated with decreased numbers of circulating T-lymphocytes and reduction of T-cell responses to antigenic stimulation (9). A number of cross-sectional studies have reported lower serum and plasma zinc levels in more advanced human immunodeficiency virus (HIV) disease (11–14), but data on copper are less consistent (15,16).

In this prospective study we examined baseline copper and zinc levels in serum, toenails, and diet in 54 asymptomatic HIV-1-seropositive homosexuals who later developed AIDS, 54 asymptomatic seropositives who remained AIDS-free, and 54 seronegatives. Our chief aims were to determine if these factors were associated with risk of progression to AIDS after adjusting for $CD4^+$ lymphocyte level and whether their levels differed between seropositives and seronegatives.

METHODS

Population and Epidemiologic Methods

The study population was identified from the Baltimore site of the Multicenter AIDS Cohort Study (MACS) (17). The Baltimore MACS is a prospective study of 1,153 (345 HIV-1 seropositive at baseline) homosexual men recruited in 1984. Participants are seen semiannually and complete a study questionnaire, receive a physical examination, and have blood drawn for hematology and storage as frozen serum and plasma. HIV-1 seropositivity is determined by a positive enzyme-linked immunosorbent assay and confirmed with Western blot testing. Percentages of $CD4^+$ lymphocytes per cubic millimeter are determined using standard flow cytometry methods. A nested case control design was used to explore the relationships between zinc, copper, HIV-1 seropositivity, and progression to AIDS. Fifty-four HIV-1 seropositives who later progressed to AIDS were matched on age (±5 years) and follow-up time with 54 seropositives who did not progress to AIDS and 54 seronegatives. Cases and controls were included in the study if, at baseline, they had complete dietary data and were free of diarrhea, weight loss, and gastrointestinal and other opportunistic infections in the previous 6 months.

Dietary intakes were determined from a 116-item version of a semiquantitative food frequency questionnaire (18) that focused on dietary intake over the preceding 12 months. The semiquantitative food frequency questionnaire includes consumption levels of foods, beverages, and nutrient supplements that are used to calculate specific nutrient intakes. The questionnaire has been widely used in epidemiologic studies and has been shown to be a reasonably valid and reliable instrument for measuring both macronutrient and micronutrient intakes (19,20). The dietary questionnaires were completed at study visit 2 in 1985, ~6 months after the collection of serum and toenail samples at study visit 1.

Laboratory Methods

Toenail specimens were cleaned by sonication in distilled water for 10 min. Samples were dried for 48 h at 37°C. Any traces of foreign material were cleaned from the nails with acetone, the nails were then rinsed and dried. Copper and zinc concentrations were determined in serial experiments beginning with copper (21).

In the first experiment, the samples were irradiated for 1 min at a thermal neutron flux of 1×10^{14} n cm^{-2} s^{-1} at the University of Missouri–Columbia Research Reactor (MURR) using the pneumatic tube facility (21). Radioactive ^{66}Cu is induced in this process [$^{65}Cu(n,\gamma)^{66}Cu$]. At the end of the irradiation, each sample was transferred to an unirradiated container and the mass was redetermined. After a 3-min decay period, the samples were real-time-counted for 10 min using a high-resolution γ-ray spectrometer. Pulse pile-up corrections were done via virtual-pulse loss-free counting using the Westphal method. The 1,039-keV γ-ray from the decay of ^{66}Cu ($t_{1/2}$ = 5.10 min) was quantitatively measured and the copper concentration was determined via standard comparison.

In the second experiment, the same samples used in the Cu determinations were transferred to a high-purity quartz vial, heat sealed, and irradiated at a thermal neutron flux of 6×10^{13} n cm^{-2} s^{-1} for ~50 h using the in-pool irradiation facility at the MURR (21). Radioactive ^{65}Zn is induced in this process [$^{64}Zn(n,\gamma)^{65}Zn$]. After a decay period of ~4 weeks, the samples were placed on an automatic sample changer and live-time counted for 2 h using a high-resolution γ-ray spectrometer. Pulse pile-up corrections were made as a part of the peak extraction process. The 1,115-keV γ-ray from the decay of ^{65}Zn was quantitatively measured and the Zn concentration was determined via standard comparison.

Serum copper concentration was determined by a Perkin-Elmer 560 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, CT, U.S.A.) (22). Serum samples of 1 ml were diluted with 3 ml of deionized water. Standards, 0–300 μg/dl, were adjusted for viscosity with 10% glycerol and for ionic strength with sodium chloride. Two quality control samples were used in the procedure, one with a mean concentration of 135 μg/dl and the other 270 μg/dl. The interassay precision was 5–6% coefficient of variation (CV). Serum zinc concentration was determined by the same absorption spectrophotometer as copper, using similar methods to plasma zinc estimations (23). Serum samples of 1.0 ml were diluted with 4 ml of deionized water. Standards, 0–200 μg/dl, were adjusted for viscosity with 10% glycerol. Two quality control samples were used in the procedure, one at 60 μg/dl and the other 120 μg/dl. The interassay precision was 5–6% CV.

Statistical Methods

Analysis of variance was used to make comparisons of micronutrient levels between the seronegatives, the seropositives who did not progress to AIDS, and the seropositives who did progress. A second analysis to determine predictors of progression to AIDS among seropositives was based on unconditional logistic regression. Independent variables were all analyzed as continuous covariates in the logistic models. Odds ratios and 95% confidence intervals are presented for incremental increases in each variable. Reported p values in post hoc t tests were not adjusted for multiple comparisons. Pearson's correlations were used to determine the relationship between dietary intakes of copper and zinc and their levels in serum.

RESULTS

The mean follow-up time in this study was 2.5 years (SD = 1.1). Mean age was 35.5 years and did not vary between the three study groups (seronegatives, seropositives who progressed, and seropositives who did not). Baseline values for copper intake, zinc intake, toenail concentrations of copper and zinc, serum copper, serum zinc, and percentage $CD4^+$ lymphocyte levels are presented in Table 1. Dietary intakes of copper and zinc did not differ significantly between HIV-1-seropositive progressors, nonprogressors, and seronegatives. Similarly, no differences in toenail levels of zinc and copper were detected between the three study groups. However, consistent significant trends were seen across all three groups in both serum copper and serum zinc levels. For serum copper, in pair-wise analyses, HIV-1 seronegatives had significantly lower baseline levels than both HIV-1-seropositive progressors ($p = 0.0001$) and nonprogressors ($p = 0.012$). For serum zinc both the seronegatives ($p = 0.009$) and seropositive nonprogressors ($p = 0.017$) had significantly higher levels at baseline than the group that progressed to AIDS. As expected, percentage $CD4^+$ lymphocyte levels were strongly associated with HIV-1 seropositivity and progression to AIDS (Table 1).

TABLE 1. *Mean micronutrient values and percentage CD4+ lymphocyte levels, at baseline, in HIV-1 seropositives who progressed to AIDS, HIV-1 seropositives who did not progress, and HIV-1 seronegatives*

	HIV+ progressors (n = 54)	HIV+ nonprogressors (n = 54)	HIV− (n = 54)	p
Copper				
Serum (μg/dl)				
Mean	115.6	109.9	101.9	0.000
SD	17.1	15.8	16.7	
Dietary (mg/day)				
Mean	4.2	3.9	4.3	0.56
SD	2.4	2.1	2.2	
Toenail (ppm)				
Mean	5.6	6.5	5.3	0.53
SD	4.4	8.2	3.2	
Zinc				
Serum (μg/dl)				
Mean	85.2	90.7	92.0	0.016
SD	11.5	12.0	14.7	
Dietary (mg/day)				
Mean	24.1	20.9	17.9	0.30
SD	22.9	25.4	10.9	
Toenail (ppm)				
Mean	102.1	104.2	103.2	0.96
SD	32.8	47.2	18.4	
Percentage CD4+ cells				
Mean	26.0	36.4	41.9	0.0001
SD	9.8	13.4	8.6	

Pearson's correlations were examined between copper and zinc intakes and their respective levels in serum and toenails. Correlations were uniformly weak and nonsignificant. Potential interactions between nutrient intakes and serum micronutrient levels were examined in stratified analyses using two-way analysis of variance. Copper intake levels were dichotomized to ≤4 and >4 mg. Copper intake did not alter the relationship between serum copper and

either HIV-1 seropositivity or progression to AIDS (for the interaction, $p = 0.61$). Zinc intake was dichotomized to ≤14- and >14-mg groups. Once again, no significant interaction was detected ($p = 0.36$). Finally, the interactions between serum copper and serum zinc with respect to HIV-1 seropositivity and progression to AIDS were also examined. The relationship of serum zinc levels to the three study groups was not altered by any interaction with serum copper ($p = 0.78$).

To determine the relative importance of serum copper and serum zinc as predictors of progression to AIDS, these variables were examined in a series of logistic regression models that adjusted for the simultaneous effects of micronutrient intake, calorie intake, percentage $CD4^+$ lymphocyte levels, age, and micronutrient levels in toenails (Table 2). The first three models include serum copper and serum zinc singly and together. Serum copper became a significant predictor of progression to AIDS after adjustment for serum zinc levels. This appears to be consistent with the previously described metabolic interactions of these two micronutrients (10). Model 4 shows that copper and zinc intake did not contribute prognostic value after adjustment for percentage $CD4^+$ lymphocyte level, but serum zinc and copper remained significantly associated with progression to AIDS. Finally, adjustment for age and inclusion of toenail zinc and copper variables did not alter the odds ratios and confidence limits obtained in model 4 (Table 2).

DISCUSSION

In this study asymptomatic HIV-1-seropositive homosexual men had higher serum copper levels and lower serum zinc levels than a similar age-matched group of HIV-1 seronegatives. Among the seropositives, those who progressed to AIDS in the next 5 years had significantly higher serum copper and lower serum zinc levels. The predictive value of these micronutrient levels persisted after adjusting for baseline percentage $CD4^+$ lymphocyte level and calorie-adjusted dietary intake of copper and zinc. There was no correlation between dietary intake of copper and zinc and the level of these micronutrients in serum. Toenail levels of copper and zinc were not related to HIV-1 seropositivity or progression to AIDS.

Zinc and copper are both known to be acute phase reactants and their levels in serum have been shown to change significantly in a range of acute and chronic infective, inflammatory, and neoplastic processes (3–8,24,25). Typically, zinc levels decrease owing largely to hepatic sequestration of zinc and to a lesser extent increased zinc excretion (6). Copper levels in serum increase in infection and neoplasia owing to increased production of ceruloplasmin (10). Our data suggest that similar changes may occur in early HIV-1 infection and appear to reflect viral activity since levels vary between HIV-1 seronegatives and seropositives and are predictive of disease progression. The lack of association of seropositivity and disease progression with nutrient intake supports the hypothesis that serum copper and zinc are markers of disease activity/progression rather than causally related. The plausibility of this hypothesis is further supported by recent data showing that viral replication can be detected in early asymptomatic HIV-1 infection (26) and that other nonspecific markers of immune system activity such as neopterin and β_2-microglobulin appear to be useful predictors of disease progression (27).

The copper and zinc responses observed in this study could also be potentially produced by opportunistic infections. This is an unlikely explanation in this study since participants who reported gastrointestinal and AIDS-related infections or disease at baseline were excluded. Thus, any acute phase response of copper or zinc is probably attributable to a direct effect of HIV-1 activity.

Cross-sectional studies have demonstrated a relationship between low serum zinc levels and late stage HIV disease (11–14), but no other longitudinal studies have been reported to date. Only one small cross-sectional study examined serum copper levels in HIV-1 infection and found raised levels in AIDS patients (16). In a larger but uncontrolled study, HIV-1-seropositive patients had either normal or high values for plasma copper (15).

TABLE 2. *Four unconditional logistic regression models of serum copper and zinc with progression to AIDS as dependent variable*

Model	Odds ratio	95% confidence interval	p
1. Serum copper (per 20 μg/dl)	1.49	1.00–2.20	0.08
2. Serum zinc (per 20 μg/dl)	0.45	0.21–0.98	0.02
3. Serum copper (per 20 μg/dl)	1.82	1.23–2.70	0.04
Serum zinc (per 20 μg/dl)	0.37	0.17–0.81	0.01
4. Serum copper (per 20 μg/dl)	2.23	1.02–4.87	0.04
Serum zinc (per 20 μg/dl)	0.30	0.14–0.66	0.01
CD4 % (per 15%)	0.30	0.17–0.54	0.001
Dietary zinc (per 10 mg)	1.03	0.85–1.25	0.73
Dietary copper (per 10 mg)	1.22	0.14–10.55	0.86

Trace metal levels in tissues such as toenails tend to reflect longer-term dietary intakes of nutrients. Thus, the lack of association between toenail copper and zinc and progression of HIV-1 infection also argues in favor of serum copper and serum zinc being risk markers rather than causally related to progression. It has been hypothesized that zinc supplementation might be useful in retarding progression of the immune deficiency in HIV-1 infection (12). Our findings do not completely exclude this possibility, but the absence of a relationship between zinc intake and progression to AIDS makes it less likely that this approach would be beneficial. This is supported by recent in vitro data showing that availability of intracellular zinc appears to be necessary for HIV-1 nucleocapsid protein assembly (28). Nonetheless, these markers appear to provide additional predictive information to $CD4^+$ lymphocyte level and may prove useful markers for clinicians and for identifying patients at similar stages of progression for clinical trials. Serum copper levels have been used as surrogate markers of response to treatment in Hodgkin's and non-Hodgkin's lymphoma (24,25) and may have similar potential in HIV infection. Further research is needed to determine more precisely what roles are played by copper, zinc, and other nutrients in the metabolic response to HIV-1 infection, as well as the potential for therapeutic intervention.

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STIC-ILL

ND 6/3

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Serum Selenium Versus Lymphocyte Subsets and Markers of Disease Progression and Inflammatory Response in Human Immunodeficiency Virus-1 Infection

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ABSTRACT

Serum selenium levels were determined cross-sectionally in 57 HIV-infected patients who were classified according to the Centers for Disease Control (CDC) 1993 classification system. Mean serum selenium levels were lower in CDC stage II (58.7 ± 12.2 μg/L; $p < 0.01$; $n = 18$) and stage III (47.6 ± 11.3 μg/L; $p < 0.01$; $n = 19$) HIV-infected patients, than in healthy subjects (80.6 ± 9.6 μg/L; $n = 48$) and stage I patients (73.6 ± 16.5 μg/L; $n = 20$). Serum selenium levels were positively correlated with CD4 count, CD4/8 ratio, hematocrit, and serum albumin ($r = 0.42$; $r = 0.39$; $r = 0.48$; and $r = 0.45$; $p < 0.01$, respectively) and inversely with serum levels of thymidine kinase ($r = -0.49$; $p < 0.01$; $n = 49$) and β2-microglobulin ($r = -0.46$; $p < 0.001$; $n = 49$). In addition, serum selenium levels in 20 randomly selected AIDS-free individuals (CDC I: $n = 10$; CDC II: $n = 10$) were inversely correlated with serum concentrations of interleukin-8 (IL-8) and soluble tumor necrosis factor receptors (sTNFR) types I and II. There was no correlation with serum immuneglobulin A and total serum protein levels. The results show that the progressive deprivation of serum selenium in HIV-infection is associated with

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loss of CD4+-cells and with increased levels of markers of disease progression and inflammatory response.

Index entries: AIDS; HIV-infection; serum selenium; thymidine kinase; β2-microglobulin; interleukin-8; soluble tumor necrosis factor receptors I and II.

INTRODUCTION

In HIV-disease the balance between pro-oxidants, antioxidants, and cytokines is disturbed toward a chronic immune activation state *(1–8)*. Oxygen radicals, hydrogen peroxide, and proinflammatory cytokines are suspected to direct intracellular signaling processes toward increased proviral transcription. It has been suggested that HIV takes advantage of the proinflammatory and prooxidative environment to replicate itself through the nuclear transcription factor κ B (NFκB) pathway *(9–12)*. Consequently, unopposed oxidative stress programs a state of steady increase of viral load. At the same time, malnutrition, owing to inadequate protein–calorie intake leads to a multilevel antioxidant deficiency that further heightens oxygen radical formation.

The essential trace element selenium acts as an integral constituent of the antioxidative enzyme glutathione peroxidase (GSH-Px, EC 1.11.1.9), which detoxifies hydrogen peroxide and organic lipid peroxides at the expense of reduced glutathione (GSH), thereby preventing the formation of the highly toxic hydroxyl radical. The oxidized form of GSH, glutathionedisulfide (GSSG), is in turn regenerated by the glutathione reductase which uses NADPH as redox equivalent, provided by the pentose phosphate shuttle. Thus, adequate concentrations of selenium and GSH are essential for optimal functioning of the GSH-Px system in order to maintain a reductive cytoplasmic milieu in HIV-infected cells to suppress the rate of HIV replication. In addition, immunological properties have been attributed to selenium *(13)*, e.g., numeric reduction of T-suppressor cells in mice *(14)*, suppression of cultured human T-suppressor lymphocytes in vitro *(14,15)*, increase of T-lymphocyte cytotoxicity and natural killer cell activities in healthy individuals *(16,17)*, and enhanced reactivity against the two recall antigens candidin and varidase and improvement of lymphocyte responses to pokeweed and phytohemagglutinin mitogen in selenium-deficient gut failure patients *(18)*.

In this preliminary study, we were interested to determine selenium levels in HIV-infected patients and correlate them with their clinical stage, with markers of disease progression and inflammatory response, such as the lymphocyte subsets, β2-microglobulin, serum thymidine kinase activity, interleukin-8 (IL-8) levels, and soluble tumor necrosis factor receptors (sTNFR) I and II *(19–22)*.

PATIENTS

Unselected HIV-infected patients (57) constituted the study group (43 outpatients, sequentially presenting to the clinic's immunological outpatient department and 14 hospitalized patients who were previously admitted to the hospital via the clinic's outpatient department). The hospitalized patients were treated for acute opportunistic infections (OI) and/or HIV-associated tumors. All patients were classified according to the Centers for Disease Control (CDC) 1993 classification system for HIV-infection (23). Characteristics of the patients are given in Table 1. Linear regression analyses were performed between serum selenium levels and markers for HIV-disease progression (CD4 count: n = 57; CD4/CD8 ratio: n = 57; β2 microglobulin, thymidine kinase activity, and immuneglobuline A: n = 49). Additionally, in 20 AIDS-free patients of the study group (CDC I: n = 10, CDC II: n = 10) serum levels of IL-8, sTNFR I and II were determined and also tested for correlation with their respective serum selenium concentrations. The subjects had not received selenium supplementation in any form for at least one year prior to selenium determination. HIV patients received standard antiviral therapy and prophylaxes appropriate to their clinical and CD4 status. The group of healthy controls consisted of 48 subjects (32 male, 16 female), average age: 40.2 yr (range: 25–55) from the region of Bonn without HIV-infection or abnormal laboratory findings.

Determination of Serum Selenium

Selenium was determined by atomic absorption spectrometry (AAS) using a 1100 Perkin Elmer atomic absorption spectrometer with a mercury hydride system (24).

Determination of T-Cell Subsets

This was done on a Becton/Dickinson-FACScan-flowcytometer using Simultest IMK-lymphocyte antibodies (CD4, CD8, CD3) from Becton/Dickinson (Heidelberg, Germany). The absolute numbers of $CD4^+$ cells were calculated on the basis of automated white blood-cell counts using an autodiffer (H1, Technicon, New York).

Serum β2-Microglobulin

This was performed on a Multiscan Titertec MCC 340 ELISA-reader, using a commercially available ELISA kit (synELISA-kit by ELIAS, Freiburg, Germany).

Table 1
Patient's Risk Factors According to the CDC 1993 Classification

Parameter	CDC I[a]	CDC II[b]	CDC III[c]
Age (yr, mean ± SD)	36 ± 11.1	37.4 ± 8.5	37.1 ± 9.1
iv Drug Use	4	5	3
Homosexual	3	6	7
Hemophiliac	2	4	5
Hetero-/bisexual	11	2	3
Blood Transfusion	—	1	1

[a] n = 20 (14 male, 6 female).
[b] n = 18 (15 male, 3 female).
[c] n = 19 (18 male, 1 female).

Thymidine Kinase Activity

This was determined in serum by radio immunoassay on a Berthold Multi crystall counter LB 2104 using a commercially available kit by Byk Sangtec (Dietzenbach, Germany).

Determination of Il-8, sTNFR I and II

The IL-8 and receptotr concentrations were determined in serum on a Multiscan Titertec MCC 340 ELISA-reader, using a commercially available ELISA kit (synELISA-kit by ELIAS).

Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA), multiple range test (Duncan) with post-hoc (Bonferoni) adjustment, and multiple and linear regression analysis using an SPSS-PC+-software package (SPSS, Inc., Chicago, IL). The data are given as mean ± standard deviation.

RESULTS

Comparison of Serum Selenium in CDC I–III Groups and Controls

Serum selenium concentrations markedly decreased with progression of HIV infection. The mean serum selenium levels of stage III (47.6 ± 11.3 µg/L) and stage II patients (58.7 ± 12.2 µg/L) were 41 and 27%, lower as compared to healthy noninfected individuals (80.6 ± 9.6 µg/L), and 35 and 20% lower as compared to CDC I (73.6 ± 16.5 µg/L) patients, respectively ($p < 0.01$). Also the mean of group II was significantly lower as compared to group III ($p < 0.01$).

Correlation of Selenium Levels with Markers of Disease Progression Marker and Immune Activation (see Figs. 1 and 2 and Tables 2 and 3)

When serum selenium levels were compared with the corresponding levels of cellular surrogate markers for progression of HIV infection, a positive correlation was found for CD4 count ($r = 0.42$; $p < 0.01$; $n = 57$) and for the CD4/CD8 ratio ($r = 0.39$; $p < 0.01$; $n = 57$). A negative correlation was found between serum selenium concentrations and activity of thymidine kinase ($r = -0.49$; $p < 0.001$; $n = 49$) and β2-microgobulin ($r = -0.46$, $p < 0.001$, $n = 49$; *see* Table 2) in serum. Multiple regression analysis of selenium, thymidin kinase, β2-microglobulin, and CD4 count revealed that only serum thymidine kinase activity was independently correlated with serum selenium, whereas CD4 count and β2-microglobulin were not independent factors with selenium in a trifactorial analysis. We found the levels of IL-8, sTNFRs I and II inversely correlated with their respective serum selenium concentrations in 20 randomly selected AIDS-free individuals ($r = 0.47$; $r = 0.5$; and $r = 0.6$; $p < 0.01$, respectively).

Serum selenium levels were positively correlated with hematocrit and serum albumin ($r = 0.48$, $p < 0.01$; $n = 57$ and 0.45; $p < 0.01$; $n = 57$, respectively) whereas not with total serum proteins or immunoglobulin A levels.

DISCUSSION

Our study shows that serum selenium levels in advanced stages of HIV disease decrease, confirming previous observations *(6,25,26,* reviewed in refs. *27* and *28–32)*. Contrary results have also been reported *(5,33)*. Factors proposed leading to selenium deficiency in HIV infection are malabsorption and loss of selenium owing to infectious diarrhea and/or HIV enteropathy, protein and caloric malnutrition. In addition, the synthesis of selenium-binding proteins, such as albumin, selenoprotein P, and GSH-Px in liver and kidney may be altered. Selenium deficiency in HIV infection, especially in active AIDS might simply reflect a state of malnutrition, which is frequently observed in severely ill patients. However, in our study, even stage II patients without obvious nutritional deficiencies had significantly lower selenium levels than asymptomatic HIV-infected individuals at stage I and healthy individuals. The lowest selenium concentrations were found in hospitalized AIDS patients, which indicates an overt marked selenium deficiency in end-stage HIV disease. An ongoing study of selenium in HIV disease in our hospital, comprising 104 patients revealed that selenium deficiency is dramatically exaggerated in the presence of an acute opportunistic infection and/or AIDS-defying tumor disease as compared to stable outpa-

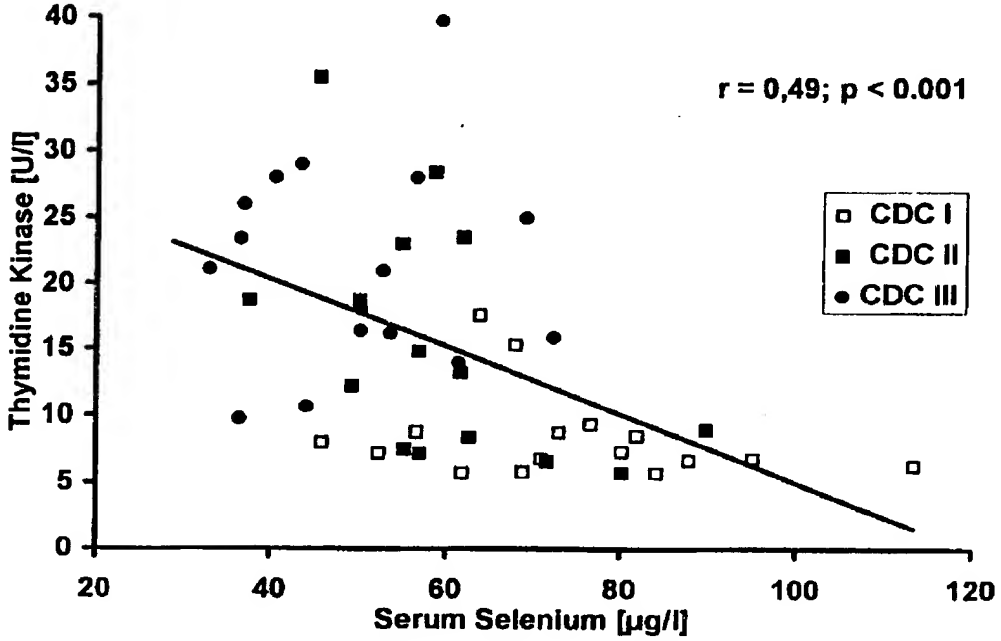

Fig. 1. Correlation of serum selenium with thymidine kinase activity in serum.

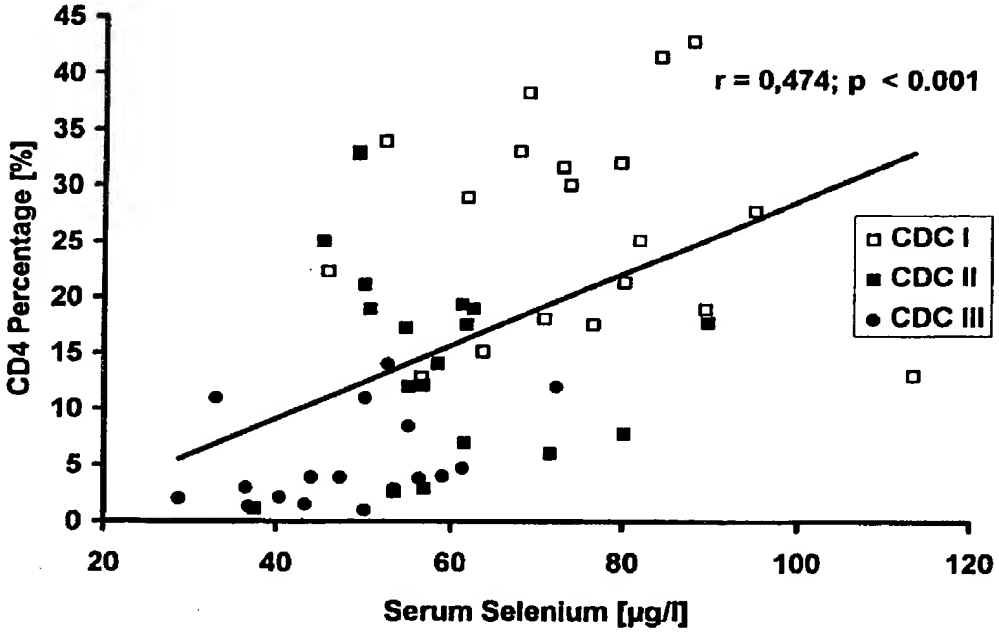

Fig. 2. Correlation of serum selenium with $CD4^+$ cells expressed as percentage of total lymphocytes.

Table 2
Serum Selenium, Biochemical Parameters, and Progression Markers of HIV Disease in CDC 1993 Groups I–III

Parameter	*n*	CDC I	*n*	CDC II	*n*	CDC III	*n*	Controls
Serum selenium (µg/L)[g]	20	73.6 ± 16.5	18	58.7[a] ± 12.2	19	47.6[a] ± 11.3	48	80.6 ± 9.6
Hematocrit (%)	20	41.8 ± 2.7	17	39.8[f] ± 4.4	16	32.1[f] ± 6.7	48	43.1 ± 3.6
Total proteins (mg/L)	20	783 ± 74	18	771 ± 67	17	725 ± 117	25	652 ± 58
Albumin (mg/L)	16	457[d] ± 49	17	444[d] ± 41	16	368 ± 52	25	430 ± 40
Lymphocytes (× 10^3 µL)	20	2273[c] ± 739	18	1354[c] ± 739	17	821 ± 901	25	1956 ± 513
CD4 cells (× 10^3 µL)	20	566.4 ± 210.8	18	226.6[e] ± 166.8	16	65.5[e] ± 130.3	25	901.6 ± 333
CD4/CD8 ratio	20	0.59 ± 0.33	18	0.33 ± 0.25	14	0.07 ± 0.05	25	1.88 ± 0.59
β2 Microglobulin (µg/L)	17	27.2[b] ± 10.5	15	33.5 ± 11.1	16	38.4 ± 12.5		not done
Thymidine kinase (U/L)	17	8.5 ± 3.4	15	14.9 ± 9.1	16	19 ± 8.9		not done

[a]Different from controls and I, $p < 0.01$.
[b]Different from III, $p < 0.05$.
[c]Different from III, $p < 0.01$.
[d]Different from III, $p < 0.05$.
[e]Different from I, $p < 0.001$.
[f]Different from controls, $p < 0.05$.
[g]One-way analysis of variance (ANOVA) + LSD test with Bonferoni adjustment.

Table 3
Correlation of HIV Disease Progression Markers with the Corresponding Serum Selenium Concentrations

Parameter	*n*	*r* vs. Selenium	Significance[a]
CD4 cells	57	0.42	$p < 0.01$
CD4/CD8 ratio	57	0.39	$p < 0.01$
Lymphocytes	57	0.34	$p < 0.05$
Hematocrit	57	0.48	$p < 0.01$
Total Proteins	57	0.24	NS
Albumin	57	0.45	$p < 0.01$
β2-Microglobulin	49	− 0.46	$p < 0.001$
Thymidine kinase	49	− 0.49	$p < 0.001$
Immunoglobulin A	49	− 0.2	NS

[a]NS = Not significant.

tients who had recovered from previous acute opportunistic infections. Furthermore, in a recent study, multivariate analysis indicated that only serum selenium levels and CD4 count were correlated with death and with the prevalence of OI, whereas p24 antigen and β2 microglobulin levels were not predictive *(34)*. In the present study, serum selenium levels were positively correlated with CD4 count, CD4/CD8 ratio, and inversely with the levels of β2-microglobulin, thymidine kinase activity, IL-8, and sTNFRs I and II. Serum IL-8 levels were shown to be elevated in HIV-infected individuals *(8)*. Its synthesis monocytes is stimulated by TNF-α. One action of IL-8 is to increase the respiratory burst in neutrophil granulocytes, thus augmenting oxidative stress. Soluble TNFRs I and II have been recently introduced as indicators of self-destructive immune activation in HIV disease *(22,35–37)*. Serum thymidine kinase levels have previously been used to detect disease progression in a number of hematological malignancies but are also helpful in the follow-up of patients with HIV infection *(20)*.

In our study, patients with elevation of serum β2-microglobulin levels above a threshold concentration of 3 µg/L were characterized by both loss of $CD4^+$ cells and selenium. Thus, the coincidence of elevated serum thymidine kinase activity, β2-microglobulin, and selenium depletion suggestes that a derangement of selenium homeostasis might occur secondary to immune activation during HIV disease progression.

Cross-sectional data, however, do not provide equivocal evidence for a cause–effect relationship between selenium deficiency and the progression of HIV disease. There is, however, experimental evidence in support of this hypothesis. It was demonstrated that GSH-Px-deficient, HIV-virus expressing cells under lipid peroxide challenge are more likely to

undergo apoptosis than noninfected control cells, or infected but non-HIV-expressing cells *(38)*. In addition, Christensen et al. *(39)* showed that selenium depletion in rats abolished GSH-Px-activity and increased the transcription of the NFκB gene. In another study, the addition of selenium (physiologic concentrations from 25–100 µg/L) to the culture medium of HIV-infected T-cells (ACH-2-cells) prior to a prooxidant/cytokine-challenge (H_2O_2/TNF-α) lead to a significant increase of GSH-Px activity and concomitantly to a reduced nuclear abundance of NFκB; this was accompanied by an increase in the number of surviving cells and inhibition of HIV-1 reactivation *(40)*.

CONCLUSION

In the light of the recent hypothesis about a putative selenium-containing HIV-repressor protein *(41)*, increased T-cell apoptosis *(42,43)*, and enhanced NFκB-mediated HIV-transcription owing to oxidative stress, inadequate selenium levels together with decreased cellular GSH concentrations erythrocyte GSH-Px activity might be considered to be potent promoters of HIV disease progression. Therefore, therapeutic strategies should aim at keeping a replete micronutrient and vitamin status *(44,45)* and suppressing oxidative stress in the early stages of HIV infection thereby reducing the proviral transcription rate.

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STIC-ILL

From: Choi, Frank
Sent: Monday, June 02, 2003 8:21 PM
To: Choi, Frank; STIC-ILL
Subject: RE: ILL_Order

-----Original Message-----
From: Choi, Frank
Sent: Monday, June 02, 2003 7:53 PM
T : STIC-ILL
Subject: ILL_Order
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Oxidative stress and plasma antioxidant micronutrients in humans with HIV infection[1-3]

Johane P Allard, Elaheh Aghdassi, Jenny Chau, Irving Salit, and Sharon Walmsley

See corresponding editorial on page 7.

ABSTRACT Increased lipid peroxidation induced by reactive oxygen species may play a role in the stimulation of HIV replication. In this study we compared lipid peroxidation indexes and plasma antioxidant micronutrients between 49 nonsmoking HIV-positive patients with no active opportunistic infection (25 asymptomatic and 24 with AIDS) and 15 age-matched seronegative control subjects. Breath-alkane output, plasma lipid peroxides, antioxidant vitamins, and trace elements were measured. Vitamin C (40.7 ± 3.02 compared with 75.7 ± 4.3 μmol/L, $P < 0.005$), α-tocopherol (22.52 ± 1.18 compared with 26.61 ± 2.60 μmol/L, $P < 0.05$), β-carotene (0.23 ± 0.04 compared with 0.38 ± 0.04 μmol/L, $P < 0.05$), and selenium (0.37 ± 0.05 compared with 0.85 ± 0.09 μmol/L, $P < 0.005$) concentrations were significantly lower in the HIV-positive patients. Lipid peroxides (50.7 ± 8.2 compared with 4.5 ± 0.8 μmol/L, $P < 0.005$), breath pentane (9.05 ± 1.23 compared with 6.06 ± 0.56 pmol · kg^{-1} · min^{-1}, $P < 0.05$), and ethane output (28.1 ± 3.41 compared with 11.42 ± 0.55 pmol · kg^{-1} · min^{-1}, $P < 0.05$) were significantly higher in the HIV-positive patients. These results showed an increase in oxidative stress and a weakened antioxidant defense system in HIV-positive patients. Whether supplementation of antioxidant vitamins will reduce this oxidative stress is still unknown. *Am J Clin Nutr* 1998;67:143–7.

KEY WORDS Lipid peroxidation, breath alkane, antioxidants, HIV infection, AIDS, oxidative stress, humans

INTRODUCTION

HIV infection induces a wide array of immunologic alterations resulting in the progressive development of opportunistic infections and malignancy, which results in AIDS. Of the mechanisms contributing to this progression, oxidative stress induced by the production of reactive oxygen species (ROS) may play a critical role in the stimulation of HIV replication and the development of immunodeficiency (1, 2).

Excessive production of ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide may be related to an increased activation of polymorphonuclear leukocytes during infections or influenced by the prooxidant effect of tumor necrosis factor α produced by activated macrophages during the course of HIV infection (3). ROS can attack double bonds in polyunsaturated fatty acids, inducing lipid peroxidation (4), which may result in more oxidative cellular damage (5, 6). Thus, measurement of lipid peroxidation is a means of determining oxidative stress. Such damage may be prevented or moderated by a normal antioxidant defense system that scavenges the ROS. This antioxidant system depends first on the integrity of an enzymatic system that requires adequate intake of trace minerals such as selenium, copper, zinc, and manganese, and second on adequate concentrations of vitamin E, A, and C and β-carotene in the cytoplasm and lipid membrane of the cells. Previous studies showed that humans infected with HIV may have deficiencies in some of these trace minerals and vitamins, such as selenium (7) and vitamin A (8).

The purpose of the present study was to measure the plasma concentrations of various antioxidants (vitamins A, C, and E and β-carotene; other carotenoids; selenium; and zinc) and some lipid peroxidation indexes in an HIV-positive population, either asymptomatic or with AIDS, and to compare these results with those in age-matched seronegative control subjects.

SUBJECTS AND METHODS

Subjects

Forty-nine HIV-positive patients [25 asymptomatic: Centers for Disease Control and Prevention (CDC) class A1 or A2; and 24 with AIDS: CDC class A3, B3, and C3] with a mean age of 39 y (range: 25–64 y) were recruited from the Immunodeficiency Clinic at The Toronto Hospital. All subjects underwent an initial screening that included a detailed history (medical, smoking, diet, and alcohol and supplemental vitamin intakes) and anthropometric (weight and height) and biochemical (complete blood count, glucose, creatinine, urea, and liver enzymes) measurements. Patients were eligible if they had no acute opportunistic infection. Exclusion criteria were as follows: smoking, initiation of antioxidant vitamin therapy before the study, hyperlipidemia, diabetes, kidney or liver dysfunction, intractable diarrhea (more than six liquid stools per day), vomiting, or evidence of gastrointestinal bleeding. Subjects were then placed

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on a controlled diet that provided a polyunsaturated to saturated fatty acid ratio of 0.3:1 for 2 wk before measurements were taken. Informed consent was obtained from all participants and the study protocol was approved by the Toronto Hospital Committee for Research on Human Subjects.

Plasma samples

Two weeks after the screening period, biochemical measurements and breath collection were performed. Blood was drawn from subjects for analysis of plasma carotenes, plasma α- and γ-tocopherol, vitamin C, lipid peroxides, selenium, and zinc.

Blood was collected into EDTA-containing tubes for the determination of carotenes, tocopherols, vitamin C, and lipid peroxides. Blood was collected into trace element–free tubes for analysis of zinc and selenium. The samples were put on ice and centrifuged promptly at 2400 rpm for 10 min at 4 °C. The plasma was removed and frozen until analyzed. Plasma for vitamin C assays was stabilized immediately with 100 g HPO_3/L (2.0 mL plasma plus 2.0 mL HPO_3).

Analyses and measurements

Breath-alkane output

Breath analysis was performed as described previously (9). Briefly, subjects were first required to breathe hydrocarbon-free air for 4 min to wash contaminating hydrocarbons from their lungs. Subsequently, expired air was collected for 2 min and analyzed by gas chromatography (Shimadzu 6-AM GC; Shimadzu Seisgkusho Ltd, Kyoto, Japan). Fifty milliliters of air was passed through a stainless steel loop packed with alumina and cooled to −95 °C to adsorb the injected sample. The loop was then heated to desorb the gas thermally. Pentane and ethane were analyzed on a Porasil D column (Chromatographic Specialties Inc, Brockville, Canada) by using a calibration curve derived from known concentrations of the gases. Concentrations of breath pentane and ethane were expressed in $pmol \cdot kg^{-1} \cdot min^{-1}$.

Lipid peroxide measurements

Plasma lipid peroxides were measured with a kit from Kamiya Biochemicals LPO (Thousand Oaks, CA). In this procedure, hemoglobin catalyzes the reaction of hydroperoxides with a methylene blue derivative, forming an equimolar concentration of methylene blue. Lipid peroxides are quantitated by calorimetry at 675 nm, and methylene blue formation is measured.

Vitamin and trace element measurements

Retinol and other carotenoids, including β-carotene, were analyzed by HPLC according to the method of Sapuntzakis et al (10). In this method, a reversed-phase C_{18} column was used with an isocratic solvent system (methanol:acetonitrile:tetrahydrofuran, 50:45:5, by vol) after hexane extraction with 200 μL serum. α- and γ-Tocopherol were analyzed with isocratic reversed-phase HPLC and fluorescence spectrophotometry at 294 nm according to the method of Nata et al (11).

Samples were analyzed for vitamin C by spectrophotometry (12). In this method, total biologically active vitamin C concentrations were determined spectrophotometrically at 521 nm with 2,4-dinitrophenylhydrazine as the chromogen.

Plasma zinc was analyzed by atomic-absorption spectrophotometry (Varian Techtron model 1200; Varian Associates, Canada Ltd, Malton, Canada) with the method described by Wolman et al (13). Plasma selenium was measured by atomic-absorption spectrophotometry at 196 nm (14). In this method, nickel salt was added as a matrix modifier to prevent volatilization of selenium during ashing.

Statistical analyses

All group data are expressed as means ± SEMs. The HIV-positive group was compared with the seronegative control subjects by using unpaired *t* tests. The minimal level of significance was identified at $P < 0.05$.

RESULTS

The HIV-positive asymptomatic patients (24 men and 1 woman) had a CD4 cell count $> 200 \times 10^9$/L and the AIDS patients (23 men and 1 woman) had a cell count $< 200 \times 10^9$/L. Most patients were receiving antiretroviral treatment (zidovudine, lamivudine, and saquinavir). Self-reported infection routes were as follows: homosexual behavior (>90%), blood transfusions, intravenous drug use, and heterosexual behavior. The control group was composed of 15 (10 men and 5 women) healthy, seronegative nonsmokers with a mean age of 35 y (range: 21–60 y) recruited from the local population, primarily hospital staff and workers from the local area. Control subjects had no acute or chronic illness and were not taking any medications or nutritional supplements.

There were insignificant differences in age (39 ± 2 y compared with 35 ± 4 y), weight (72.1 ± 2.4 kg compared with 71.3 ± 2.0 kg), and body mass index (in kg/m^2) (23 ± 1 compared with 24 ± 1) between the HIV-positive patients and control subjects, respectively. HIV-positive patients had significantly lower antioxidant vitamin concentrations (vitamin C, α-tocopherol, and β-carotene) than control subjects (**Table 1**). Other carotenoids, such as lycopene, β-cryptoxanthin, and lutein/zeaxanthin were also significantly lower in the plasma of the HIV-positive group than in the control subjects (**Table 2**).

Lipid peroxidation determined from breath-alkane output and plasma lipid peroxide concentrations were significantly higher in the HIV-positive group than in control subjects (**Table 3**). There were no significant differences in lipid peroxidation or plasma antioxidant vitamins between HIV-positive asymptomatic subjects and those with AIDS (data not shown).

TABLE 1
Plasma antioxidant vitamins in HIV-positive subjects and seronegative control subjects[1]

	HIV-positive patients ($n = 49$)	Control subjects ($n = 15$)
α-Tocopherol (μmol/L)	22.52 ± 1.18	26.61 ± 2.6[2]
γ-Tocopherol (μmol/L)	2.69 ± 0.21	7.65 ± 0.80[3]
Vitamin C (μmol/L)	40.7 ± 3.02	75.7 ± 4.3[3]
β-Carotene (μmol/L)	0.23 ± 0.04	0.38 ± 0.04[2]
Retinol (μmol/L)	2.03 ± 0.09	2.11 ± 0.12
Zinc (μmol/L)	15.45 ± 0.92	14.53 ± 1.53
Selenium (μmol/L)	0.37 ± 0.05	0.85 ± 0.09[3]

[1] $\bar{x}$ ± SEM.
[2,3] Significantly different from HIV-positive patients: [2] $P < 0.05$, [3] $P < 0.005$.

TABLE 2
Other plasma carotenoids in HIV-positive subjects and in seronegative controls subjects[1]

	HIV-positive patients (n = 49)	Control subjects (n = 15)
α-Carotene (μmcl/L)	0.061 ± 0.009	0.115 ± 0.035
Lutein ± zeaxanthin (μmol/L)	0.19 ± 0.02	0.35 ± 0.03[2]
β-Cryptoxanthin (μmol/L)	0.09 ± 0.01	0.16 ± 0.03[3]
Lycopene (μmol/L)	0.32 ± 0.04	0.64 ± 0.07[2]

[1] $\bar{x}$ ± SEM.
[2,3] Significantly different from HIV-positive patients: [2] $P < 0.005$. [3] $P < 0.05$.

DISCUSSION

The results of this study showed that in this group of HIV-positive subjects, oxidative stress was significantly higher than in seronegative control subjects as determined from breath-alkane output and plasma lipid peroxide concentrations. There were no significant differences in lipid peroxidation between asymptomatic patients and those with AIDS. The increase in lipid peroxidation was also associated with lower plasma concentrations of antioxidant micronutrients such as vitamin C, α-tocopherol, β-carotene, and selenium.

Breath-alkane output was also studied by our group in previous studies (15, 16). Breath pentane and ethane evolve from the peroxidation of n−6 and n−3 fatty acids, respectively. These volatile hydrocarbon gases are produced by the β-scission of polyunsaturated fatty acids and are passed from the lungs into the expired air (9). In human studies, measurement of these alkanes in the breath is noninvasive and has been used and validated as a measure of lipid peroxidation (9, 17, 18). Intake of n−3 and n−6 fatty acids can influence the composition of the alkane produced (pentane and ethane) (19). For this reason, the subjects were given instructions about their dietary fat intake 2 wk before the measurements. Smoking (20), liver disease (21), and alcohol consumption (22) can also affect breath-alkane output. The subjects were screened before being enrolled and excluded if these confounders were present.

The finding of increased lipid peroxidation by this method is consistent with the finding of other studies that showed oxidative stress in HIV-positive patients (23–26) as evidenced by plasma lipid peroxide and malondialdehyde concentrations. Increased breath-pentane output was also reported in a small number of HIV-positive patients (25). Another index of lipid peroxidation, malondialdehyde concentrations, was also significantly higher in 26 asymptomatic HIV-positive patients (stage II) than in seronegative control subjects, and even higher in patients with AIDS (24). However, in our study we did not detect a significant difference between asymptomatic HIV-positive patients and those with AIDS.

The mechanisms underlying the increased oxidative stress in the HIV population remain unclear. In addition to an excessive production of ROS, which may be explained by polymorphonuclear leukocyte activation during infectious c nditions or by a prooxidant effect of tumor necrosis factor α produced by activated macrophages (3), a weakened antioxidant defense system may play a role. To our knowledge, our study is the first to document significantly higher oxidative stress and lower concentrations of major plasma antioxidants (ascorbic acid, α-tocopherol, β-carotene, and selenium) in the same HIV-positive subjects than in seronegative control subjects.

Another study by Constans et al (27) also showed that HIV-positive patients had significantly lower plasma vitamin A and selenium concentrations than control subjects and showed that alterations in these antioxidants were correlated with a decrease in polyunsaturated fatty acids, a target of free radicals. Another study (28) reported significantly higher superoxide dismutase and glutathione peroxidase activities in HIV-positive patients than in uninfected control subjects. There was no significant effect of selenium or β-carotene supplementation on superoxide dismutase activity compared with baseline but glutathione peroxidase activity and glutathione status increased. Because it is known that glutathione peroxidase plays a central role in the metabolism of ROS, this study suggests that antioxidant supplements may have an effect on oxidative stress in HIV-positive patients. This could be of great interest because deficiencies in antioxidant vitamins and trace minerals are common in HIV infection, especially in advanced stages of the disease (29–33). In fact, in HIV-positive patients plasma and red blood cell selenium concentrations (29) and glutathione concentrations were found to be low (30, 31). Deficiencies in antioxidant vitamins were also reported for vitamins E (32) and C (33).

This antioxidant deficiency in HIV-positive populations is probably due to increased utilization of antioxidant micronutrients because of increased oxidative stress rather than to inadequate dietary intake (34) or malabsorption (35). However, although most of the HIV-positive individuals in the study by Baum et al (36) generally consumed dietary amounts that were equal to or greater than the recommended dietary allowance (RDA) (eg, for vitamins B-6, B-12, and A), other micronutrients such as vitamin E and zinc generally were consumed in amounts less than the RDA. Another study also showed that reported intakes of various micronutrients from food exceed 100% of the RDA, except for energy, zinc, thiamine, and vitamin E (37). Our own preliminary data from 3-d food records collected from the population studied indicated that dietary macro- and micronutrient intakes were equivalent to the RDA at the time of the study

TABLE 3
Lipid peroxidation indexes in HIV-positive subjects and seronegative control subjects[1]

	HIV-positive patients (n = 49)	Control subjects (n = 15)
Breath-pentane output (pmol·kg^{-1}·min^{-1})	9.05 ± 1.23	6.06 ± 0.56[2]
Breath-ethane output (pmol·kg^{-1}·min^{-1})	28.1 ± 3.41	11.42 ± 0.55[2]
Plasma lipid peroxide (μmol/L)	50.7 ± 8.2	4.5 ± 0.8[3]

[1] $\bar{x}$ ± SEM.
[2,3] Significantly different from HIV-positive patients: [2] $P < 0.05$. [3] $P < 0.005$.

(data not shown). Because our HIV population had a normal nutritional status, on the basis of body mass index, we speculate that malabsorption was not significant. Thus, it is possible that the reduced plasma antioxidant concentrations observed in our population were due to an increased consumption of these micronutrients secondary to chronic oxidative stress from infection. A weakened antioxidant defense system, in turn, could lead to further enhancement in lipid peroxidation.

This increase in oxidative stress documented in our HIV-positive population may have some clinical significance because there is experimental evidence implicating oxidative stress in the stimulation of HIV replication. In vitro experiments (2) have shown that ROS such as hydrogen peroxide can specifically activate the nuclear factor κB to induce the expression and replication of HIV-1 in a human T cell line, and addition of antioxidant vitamins blocked activation of nuclear factor κB and inhibited HIV replication (38–40). Although observational studies suggest that an increased intake of some antioxidants may delay progression to AIDS (37, 41), eg, > 750 mg vitamin C/d, > 130 mg vitamin E/d, and > 7243 RE (retinol equivalents) carotenoids/d (37), no clinical trials have investigated the effect of antioxidant supplementation on oxidative stress and viral load.

In conclusion, this study showed that lipid peroxidation, measured by breath-alkane output and lipid peroxide concentrations, was significantly higher in HIV-positive patients than in seronegative control subjects, and plasma concentrations of various antioxidant vitamins and selenium were significantly lower. These results along with the findings reported in the literature suggest that a weakened antioxidant defense system may play a significant role in the increased oxidative stress found in this population, whether due to increased consumption or reduced intakes of antioxidant micronutrients. However, it remains to be determined whether antioxidant supplementation will have any effect, not only on oxidative stress but also on viral replication and disease progression.

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ORIGINAL ARTICLE

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Improvement of immune functions in HIV infection by sulfur supplementation: Two randomized trials

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Abstract To determine the therapeutic effect of sulfur amino acid supplementation in HIV infection we randomized 40 patients with antiretroviral therapy (ART; study 1) and 29 patients without ART (study 2) to treatment for 7 months with *N*-acetyl-cysteine or placebo at an individually adjusted dose according to a defined scheme. The main outcome measures were the change in immunological parameters including natural killer (NK) cell and T cell functions and the viral load. Both studies showed consistently that N-acetyl-cysteine causes a marked increase in immunological functions and plasma albumin concentrations. The effect of *N*-acetyl-cysteine on the viral load, in contrast, was not consistent and may warrant further studies. Our findings suggest that the impairment of immunological functions in HIV$^+$ patients results at least partly from cysteine deficiency. Because immune reconstitution is a widely accepted aim of HIV treatment, *N*-acetyl-cysteine treatment may be recommended for patients with and without ART. Our previous report on the massive loss of sulfur in HIV-infected subjects and the present demonstration of the immunoreconstituting effect of cysteine supplementation indicate that the HIV-induced cysteine depletion is a novel mechanism by which a virus destroys the immune defense of the host and escapes immune elimination.

Key words HIV infection · Viral load · Immune reconstitution in HIV infection · NK cell activity · *N*-Acetyl-cysteine

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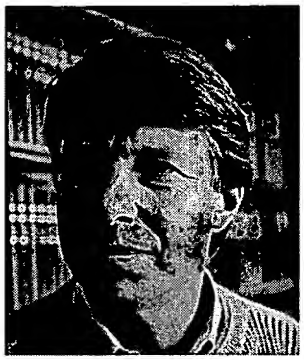

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Abbreviations *ART:* Antiretroviral therapy · *GSH:* Glutathione · *HAART:* Highly active antiretroviral therapy including a protease inhibitor · *HIV:* Human immunodeficiency virus · *IL-6:* Interleukin-6 · *NAC: N*-Acetyl-cysteine · *NK:* Natural killer · *PE:* Point estimate of shift

Introduction

Previous studies on macaques infected with simian immunodeficiency virus have revealed markedly increased intracellular sulfate and decreased glutathione (GSH) levels in the skeletal muscle tissue, suggesting that this retroviral infection causes an increase in the muscular cysteine catabolism at the expense of the cysteine and GSH pools [1]. In line with this notion, we and others [2, 3, 4, 5, 6, 7] have shown that patients infected with human immunodeficiency virus (HIV) have abnormally low plasma cystine concentrations and low GSH levels, although some authors have not observed decreased GSH levels in peripheral blood cells [8, 9]. A more recent study revealed that the peripheral tissue of HIV$^+$ patients with or without highly active antiretroviral therapy (HAART) releases large amounts of sulfate that would account for a loss of approximately 5 g cysteine per day if extrapolated to a person of approximately 70 kg body weight [10]. A complementary investigation on 64 *asymptomatic* HIV$^+$ patients and 65 HIV$^-$ subjects revealed increased plasma sulfate levels in the HIV$^+$ patients.

The analysis of the daily urinary excretion of sulfate and urea of HIV$^+$ patients and healthy HIV$^-$ subjects confirmed (a) that HIV$^+$ patients experience a massive loss of sulfur, and (b) that this loss is not ameliorated by (HA)ART. The net loss of sulfur in asymptomatic patients was equivalent to a *net* loss of about 7 g cysteine per day [10]. If extrapolated, this would correspond to an alarming negative balance of approximately 2 kg cysteine per year under the assumption that the *normal* sulfate excretion equivalent to approximately 3 g per day is balanced by a standard Western diet. The abnormally high sulfate/urea ratio suggested that this process drains largely the GSH pool [10]. To ameliorate the immunological consequences of the virus-induced cysteine and GSH deficiency in HIV infection we have previously proposed treatment with *N*-acetyl-cysteine (NAC) [11, 12, 13]. In the meantime, several clinical studies on the effects of NAC have been performed [14, 15, 16, 17, 18, 19]. These results of these studies have in general been promising, but they are controversial because the doses of NAC were relatively low, or the observation period was relatively short [15, 16, 17, 18, 19]. One investigation [14] with relatively high doses suggested that NAC improves the 2-year survival rate, but this investigation was not randomized, and the doses were chosen arbitrarily and may have been too high for some patients, for reasons discussed below. A placebo-controlled randomized study on the effects of NAC on immunological functions and virus load has not been described previously.

Our two randomized studies on HIV$^+$ patients with and without ART involved 7 months of treatment with individually adjusted doses of NAC. Outcome measures included NK cell activity and several proliferative T cell responses. These immunological functions are decreased relatively early in HIV infection [20, 21, 22, 23, 24]. Several reports suggest that NK cells play a protective role in HIV infection [25, 26, 27, 28]. Additional outcome measures were viral load, CD4$^+$, CD8$^+$ and CD16$^+$/CD56$^+$ cell numbers, body cell mass, plasma albumin, interleukin-6 (IL-6), thioredoxin and glutamine levels. Plasma glutamine is decreased relatively early in HIV infection [29], and a decrease in the extracellular glutamine level impairs lymphocyte functions [30, 31, 32, 33].

Patients and methods

Double-blind randomized clinical trial on the effect of NAC in combination with antiviral therapy (study 1)

Clinically asymptomatic HIV$^+$ patients (CDC I, 1993, age over 18 years) with 200–500 CD4$^+$ cells/mm^3 were eligible as determined less than 2 months before recruitment. Exclusion criteria were endocrinological diseases, liver cirrhosis, serum creatinine higher than 1.5 mg/dl, cardiorespiratory insufficiency, alcohol or drug abuse. Female patients had to use contraception throughout the study. At study entry the clinical laboratory data were in the normal range. All patients had been under constant ART for at least 6 months before and throughout the study (Table 1). The trial profile is shown in Fig. 1. Block randomization (individual) was performed by the Biostatistics Unit of the German Cancer Research Center (L.E.). The code was known only to L.E. After generation of the assignment by L.E., the patients were recruited, and the blinded assignment was executed by R.B. at the clinical centers in Mannheim. The code for the assignment to one or another group was broken after completion of the last measurement. The code for the assignment to verum vs. placebo was broken after completion of the statistical analysis. The study was approved by the ethics committee of the Medical Faculty of the University of Heidelberg/Mannheim and was conducted according to the guidelines of good clinical and laboratory practice and the principles of the Declaration of Helsinki.

The patients were examined before the start of therapy within 3 days after informed consent and 4 weeks, 4 months, and 7 months later. NAC (600 mg tablets or placebo with same taste) was administered every second day during this period. Empty blisters were returned by the patients to the physicians to confirm compliance. The initial daily dose was 3.6; 2.4, 1.2, and 0.6 g for patients with plasma glutamine levels lower than 450, 450–500, 500–600, and higher than 600 µM, respectively. After the subsequent examinations the dose was either increased by 2.4, 1.8, 1.2, not changed, decreased to half of the previous dose, or decreased to 0.6 g/day if the patients had plasma glutamine levels lower than 450, 450–500, 500–600, 600–700, 700–800, or 800–900 µM, respectively.

Double-blind randomized clinical trial on the effect of NAC without antiviral therapy (study 2)

Eligible were clinically asymptomatic ART-naive HIV$^+$ patients (CDC I, 1993) with more than 200 CD4$^+$ cells/mm^3 as determined less than 2 months before recruitment. For other details see study 1.

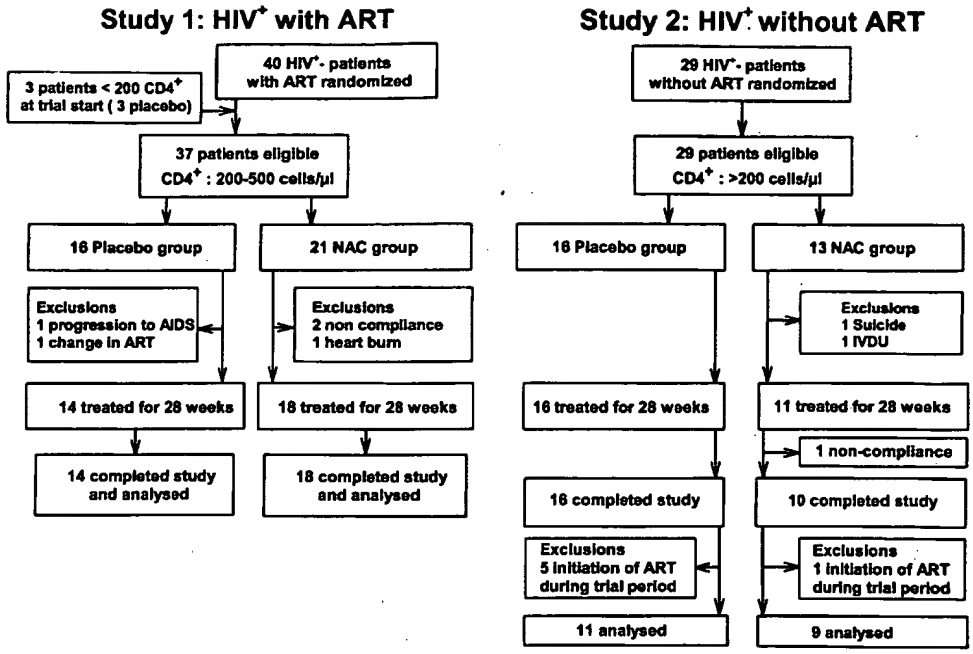

Fig. 1 Trial profiles of studies 1 and 2

Sample size

The two studies were originally planned as a single study with two strata defined by different inclusion criteria. A sample size of n=30 per treatment group was calculated with the aim to detect a significant difference in the treatment group of the combined strata at the significance level of 5% by a one-tailed paired t test with a power of 80%. In the absence of information about the range and variability of the viral load and immunological parameters in patients with and without NAC treatment, we determined that sample size on the basis of plasma cystine levels such that an increase in the cystine level by 10.0 µM after two cycles with a standard deviation of 15 µM was detectable. A drop-out rate of 10–20% was assumed. In order to adjust for multiple endpoints of the plasma cystine level and other variables a maximum of 14 comparisons was considered, and the error per experiment was set to 0.05/15=0.0035 to achieve a multiple significance level of 0.05. The same sample size of n=30 was planned for the placebo control.

Physical, biochemical, and functional tests

Plasma amino acids were determined as described [3, 4, 5, 29] using postabsorptive blood samples from the cubital vein. The intra-assay and inter-assay variation (100×SD/mean, n=20) of the glutamine assay was 0.83% and 2.80%, respectively. The HIV RNA levels were determined by the HIV-1 QT Nuclisens-test kit (Organon Teknika) [34, 35] and cell types by three-color staining with CD3, CD4, CD8, CD14, CD16, CD19, CD45, and CD56 antibodies (Becton Dickinson/Simultest) on a FACScan. The proliferative responses against phytohemagglutinin and immobilized CD3 plus CD28 antibodies were determined as described [24] with minor modifications. Specifically, peripheral blood lymphocytes were incubated for 3 days without and another 18 h with [^{3}H]thymidine prior to harvesting. The response against tetanus toxin (Behring, Marburg, Germany) was determined as described [22] with an incubation period of 7 days plus 18 h. The NK cell activity was determined as described [21]. For comparison and validation of the assays, all patient samples were run in parallel to at least one healthy donor sample on the same day (see following section). Plasma thioredoxin and albumin levels were determined as described [36, 37], and IL-6 was assayed by the IL-6 EASIA test kit (Biosource, Ratingen, Germany) [38].

HIV$^-$ control group

NK cell activities, cell types, proliferative activities, plasma amino acids, and albumin levels were determined during the trials also in 87, 56, 74, 107, and 42 HIV$^-$ control subjects, respectively (see Fig. 2).

Statistical analysis

With the exception of Table 2, our analysis was carried out "per protocol" (i.e., by "as treated analysis") to most closely reflect the scientific model underlying the protocol. The baseline data of the drop-outs (Table 1) suggest no bias of this procedure compared with an "intent-to-treat analysis." The statistical analysis was performed under blinded conditions. The relative changes in outcome parameters of the two different treatment groups were compared by the Wilcoxon rank-sum test (Figs. 4, 5), and individual changes between baseline and terminal examinations in each treatment group were analyzed by the Wilcoxon sign-rank test (Fig. 3). P values <0.05 were regarded as statistically significant. Hodges-Lehmann point estimate shifts (PE) with exact 95% confidence intervals, arithmetic or geometric means, and standard errors of the mean together with graphic box plots were used as descriptive statistics. The "intent-to-treat analysis" of the main parameters is shown in Table 2.

Results

Baseline data from studies 1 and 2 and control data from HIV$^-$ subjects

The baseline data of the two studies were comparable in the respective treatment groups before the start of the trial (Table 1). The NK cell activity, stimulation indices, plasma glutamine, and albumin levels from all groups of HIV$^+$ patients were abnormally low in comparison with HIV$^-$ subjects regardless of whether the patients had been treated with or without ART or with highly active

Table 1 Baseline characteristics of studies 1 and 2 (*SI* stimulation index)

	Study 1		Study 2	
	NAC group (n=21)	Placebo group (n=16)	NAC group (n=13)	Placebo group (n=16)
Males/females	11/10	10/6	8/5	8/8
Monotherapy with Nucleoside analogs (n)	2 (ddI/AZT)	1 (ddI)	0	0
Two nucleoside analogs (n)	12	10	0	0
HAART (n)	7	5	0	0
Mean age (years)	39.7±2.4	40.1±2.6	32.6±2.3	39.2±2.9
$CD4^+$ cells (mm^{-3})	367±32	366±37	503±70	491±56
Virus load (log copies/ml)	4.1±3.62	3.6±3.2	4.7±4.51	4.4±3.9
NK (lytic units per 10^7 cells)	31.6±9.7	58.8±15.6	43.9±11.5	46.6±9.9
NK (lytic units per $CD3^-/CD16^+/CD56^+$ cell)	0.22±0.06	0.43±0.13	0.33±0.13	0.54±0.20
SI αCD3/CD28	364±73*	1045±296*	785±164	568±107
SI phytohemagglutinin	746±137	909±264	779±163	996±138
SI tetanus toxin	25±14	41±18	40±30	35±13
Plasma albumin (μM)	637.7±13.7	650±13.1	573.4±33.9	584.4±25.9
Plasma IL-6 (ng/ml)	44.4±5.9	31.0±5.3	54.1±4.2	64.6±7.0
Plasma glutamine (μM)	568.9±18.6	577.1±16.9	585.5±32.2	570.6±24.0
Plasma arginine (μM)	47.3±3.9	56.1±6.8	59.7±5.8	60.3±4.5
Plasma cystine (μM)	42.0±2.1	41.2±2.9	38.6±2.3	45.7±2.5

*P=0.03 for the difference between the corresponding NAC and placebo groups

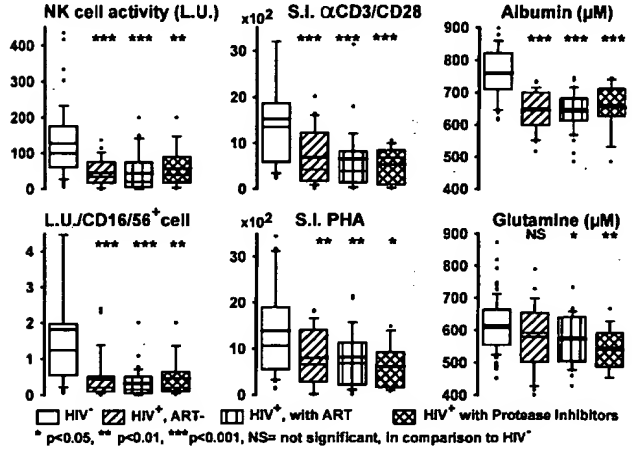

Fig. 2 Baseline characteristics of HIV^+ persons with and without ART and data from HIV^- subjects. The four columns represent the HIV^- control group (see text), ART-naive HIV^+ persons (baseline data of study 2), patients with ART (baseline data of study 1) and patients from study 1 treated with HAART (i.e., a protease inhibitor plus one or two nucleoside analogs; n=12). *Box plot* describe the first (25%) and third (75%) quartile of the distribution, the arithmetic mean (*strong line*) and the median (*thin line*). The NK cell activity is expressed as lytic units per 10^7 peripheral blood mononuclear cells (*upper left panel*) or as lytic units per $CD16^+/CD56^+$ cell

antiretroviral therapy (HAART) involving at least one protease inhibitor (Fig. 2).

NAC treatment with individually adjusted doses

The plasma glutamine level was used as a guideline against NAC overdosing because an excessive cysteine catabolism in the liver is associated with the production of protons and may inhibit urea production in favor of glutamine production eventually to the point that the glutamine forming capacity is exceeded, and toxic ammonia accumulates [39]. In pilot investigations on several NAC-treated HIV^+ patients we occasionally observed abnormally high plasma glutamine levels (>900 μM) and cystine levels (>150 μM: W.D., unpublished observations). As a rule, the plasma glutamine level was found to increase or decrease whenever the dose of NAC was increased or decreased, respectively, as shown by the example in Fig. 3A. We therefore did not give a constant dose of NAC to all patients, but decreased the dose of NAC whenever the plasma glutamine level exceeded 700 μM (see protocol). The mean dose of NAC that was administered according to the defined protocol every second day during the three intervals of the observation periods is shown in Fig. 3B and C and was about 3 g NAC at the end of the trials.

The effects of NAC in combination with or without ART: Reconstitution of immunological and biochemical parameters and effect on viral load

NAC caused a strong consistently increase in all immunological test parameters in both studies (Fig. 4, NK cell activity in study 1: PE 732.8, 95% CI 212–2128; study 2: PE 363.1, 95% CI 64–2185; for units see legend to Fig. 4). The analysis on the basis of "intent-to-treat" revealed in the case of study 1 identical results and in the case of study 2 similar results. Table 2 shows the "intent-to-treat" analysis of the most relevant parameters. The NK cell activity of NAC treated patients increased to almost normal levels (Fig. 3). The changes in $CD4^+$ (Table 2) and $CD8^+$ cell numbers and plasma thioredoxin levels (not shown), in contrast, did not differ significantly between the treatment groups in the two studies.

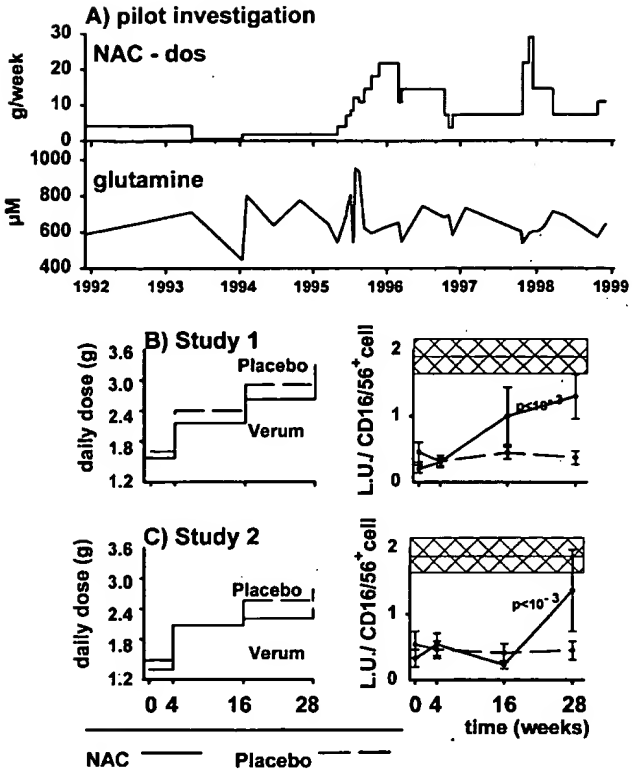

Fig. 3A–C Longitudinal changes in HIV-infected individuals during a NAC treatment. **A** Pilot investigation. A 50-year-old male HIV-infected healthcare worker with a $CD4^+$ cell count of 175/mm³ and without clinical symptoms started to take NAC (0.6 g/day) in 1991. As a rule, the patient received the minimum dose of NAC to maintain plasma glutamine at a level higher than 600 µM (i.e., the mean of healthy subjects). From 15 December 1995 the patient also received ART. At the time of submission of this report, the patient is still symptom-free. **B** Study 1. *Left* the means of the doses of NAC or placebo that were administered every second day according to the treatment scheme. The NK cell activity is given as lytic units per $CD3^-/CD16^+/CD56^+$ cell. *Error bars* SEM; *shaded area* mean ±SEM of HIV^- persons (see description of HIV^- control group in text). *P* values refer to differences between terminal and baseline examination. **C** Study 2. For details see **B**

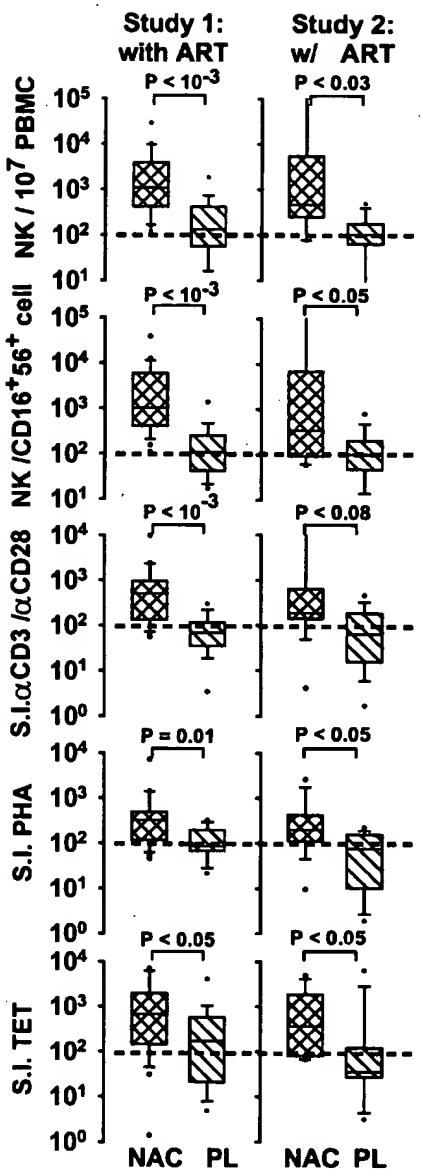

Fig. 4 Effect of NAC on the relative changes in NK cell activity and T cell stimulation indices. Median and box plots of the relative changes in NK cell activity per 10^7 peripheral blood mononuclear cells, NK cell activity per $CD3^-/CD16^+/CD56^+$ cell, and the relative changes in the stimulation indices in response to immobilized CD3 plus CD28 antibodies, phytohemaglutinin (*PHA*) and tetanus toxin (*TET*) from single individuals at the last examination (i.e., after 7 months of therapy) expressed as percentage of the corresponding individual baseline values. *P* values are given for the difference between the NAC and placebo group. *Dashed line* no change (100%). The effects of NAC were not detectable after 4 weeks but were already visible after 4 months of therapy (not shown)

The placebo group of study 1, i.e., patients who received stable ART for more than 6 months before plus 7 months throughout the study showed on average a significant increase in viral load during the observation period (P=0.01 for the longitudinal change within the group) indicative of ART failure (Fig. 5, Table 2). This increase was not seen in the NAC-treated group (PE 152.0, 95% CI –22.7 to –432.1, P=0.014 for the comparison between treatment groups). However, in view of the differences between the mean virus loads of the NAC group and the placebo group at baseline examination, the differential increase in virus load may be explained by the higher baseline level in the NAC-treated group rather than by the effect of NAC (Table 2). Also, there was no significant effect of NAC on the virus load in study 2.

Finally, NAC increased the mean plasma glutamine level (PE 16.0, 95% CI 6.0–26.0, P<0.01, data not shown) and reversed the decrease in plasma albumin (PE 14.3, 95% CI 9.15–19.43) and the increase in IL-6 (PE –38.7, 95% CI –21.8 to –56.3) in the ART-treated patients (Fig. 5, study 1). These effects were not statistically significant in study 2, but the increase in plasma albumin and decrease in IL-6 were significant (P<0.001 and

Table 2 Treatment effects in the two trials by Intent to treat analysis (*SI* stimulation index)

	Study 1				Study 2			
	NAC		Placebo		NAC		Placebo	
	Baseline	Terminal	Baseline	Terminal	Baseline	Terminal	Baseline	Terminal
NK (Lytic units per $CD3^-/16^+/56^+$ cell)	0.22±0.06	1.34±0.34	0.45±0.15	0.37±0.26	0.33±0.13	1.33±0.56	0.52±0.19	0.47±0.11
SI PHA	747±137	1906±355	967±292	759±131	779±164	1267±301	1022±132	906±221
SI TET	25±14	150±128	43±21	37±23	40±31	190±139	46±17	31±15
Virus load	4.10±3.62	4.08±3.55	2.28±0.51	3.53±0.19	4.17±0.24	3.74±0.24	3.82±0.22	3.40±0.25
$CD4^+$ cells (mm^{-3})	367±32	432±53	365±39	421±39	503±71	513±77	476±55	437±42

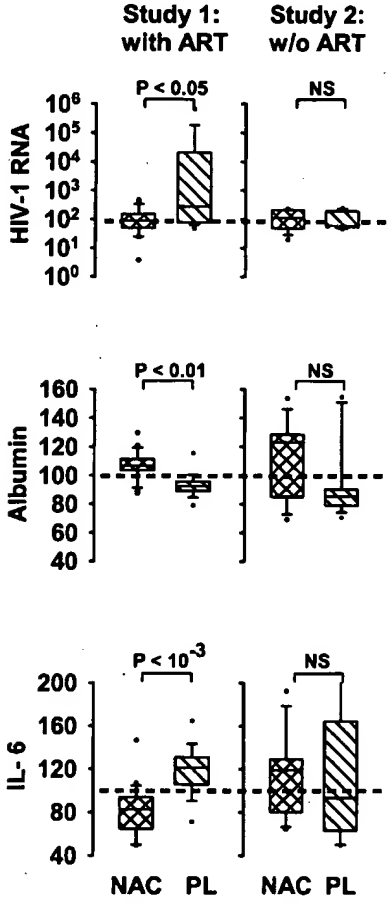

Fig. 5 Effect of NAC on the relative changes in virus load, plasma albumin, and plasma IL-6 levels. Median and box plots of the measurements. For other details see legend to Fig. 4

$P<0.05$, respectively), when both studies were taken together.

There were no adverse events except for two NAC-treated subjects with heart burn in study 1. In one case this disappeared spontaneously. The other patient had to be excluded from the study.

Discussion

Because immune reconstitution is a major goal of HIV therapy, the consistent improvement in several immunological functions in our two randomized studies on NAC strongly suggests that HIV-infected patients with or without ART or HAART should receive NAC treatment. There is an increasing awareness that current antiretroviral drugs can decrease HIV replication and virus load considerably but fail to abolish virus replication completely [40, 41]. A proportion of patients show an increase in HIV RNA levels in spite of ART, i.e., a condition that is commonly interpreted as ART failure [40] (see also study 1). The significant beneficial effects of NAC even in the group of patients who already received (HA)ART were in line with a recent study on the daily urinary sulfate excretion which revealed (a) that HIV^+ patients experience a massive loss of sulfur, and (b) that this loss is *not* ameliorated by (HA)ART [10]. Moreover, in spite of the indisputable merits of HAART it should be noted that ART without protease inhibitors is still the treatment of choice for many HIV^+ patients who cannot afford or tolerate HAART.

The immune reconstitution by NAC is also in line with a previous nonrandomized investigation which provided suggestive evidence that NAC treatment may increase the 2-year survival rate [14]. Because the dose was not adjusted to individual needs in this earlier study, we have reason to believe that those results could still be improved considerably. Because the patients require on average rather large doses of NAC, we propose that the individual dose of NAC should be decreased whenever the plasma glutamine (or the albumin level) is in the upper normal range. The effect of NAC on the plasma albumin level (Fig. 5) confirmed earlier findings in cancer patients [37] and is best explained by the fact that the oxidized form of albumin has a faster clearance rate [37]. This interpretation is in agreement with earlier reports that the rate of albumin synthesis was *increased* in several conditions of infection and trauma even when albumin concentrations were *decreased* [42, 43]. Earlier studies on smaller numbers of HIV-infected patients did not show significantly decreased plasma albumin concentrations but increased plasma concentrations and synthetic rates of "positive" acute-phase proteins [43, 44]. Wheth-

er NAC treatment may have a general anti-inflammatory effect and reverse the increase in acute-phase proteins remains to be determined.

Because several studies on HIV$^+$ patients and simian immunodeficiency virus infected monkeys collectively suggest that HIV/simian immunodeficiency virus infection may increase the cysteine catabolism and deplete the cyst(e)ine and GSH pools (see "Introduction"), we propose tentatively that the therapeutic effect of NAC reflects the reconstitution of the cysteine deficiency rather than a general immunopotentiating effect of NAC. If so, NAC is exceptional among drugs for the acquired immunodeficiency syndrome. There is a strong possibility that the immune system is among the first to suffer from this excessive loss of cysteine. Because GSH and cysteine have been shown to regulate the activation of the transcription factor nuclear factor-κB and the nuclear factor-κB-dependent replication of HIV [45, 46], it may be tempting to speculate that the beneficial effects of NAC are due to the inhibition of nuclear factor-κB and viral replication. However, we did not see a significant effect of NAC treatment on HIV RNA levels. In view of the decreased GSH levels in peripheral blood mononuclear cells of HIV$^+$ patients [3, 14], it may be tempting to explain the immunological effects of NAC by its function as a GSH precursor. This question has not been addressed here. NAC has failed, however, in several earlier studies to increase GSH levels of mononuclear cells in vivo [37, 47, 48, 49] but did increase the GSH level in the liver [50, 51] and erythrocytes [14, 18]. Studies on the venous plasma concentrations of NAC have led others to conclude that the oral bioavailability of NAC is low [19, 52], but these authors obviously dismiss the direct uptake and storage of cysteine by the liver from the portal vein. Magnusson and colleagues [53] reported the deacetylation and the subsequent release of cysteine into the blood plasma by the kidneys after intravenous infusion of NAC. Our placebo-controlled trials of NAC treatment clearly indicate that NAC may be beneficial in the treatment of HIV-infection and may reverse the decrease in immunological reactivity. The HIV-induced excessive loss of cysteine and the reconstitution of immunological functions by NAC indicate, finally, that the virus-induced cysteine deficiency may be a novel mechanism by which a virus destroys the immune defense of the host and avoids its elimination by the immune system.

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Sent: Monday, June 02, 2003 8:21 PM
To: Choi, Frank; STIC-ILL
Subject: RE: ILL_Order

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To: STIC-ILL
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Status of selected nutrients and progression of human immunodeficiency virus type 1 infection[1–4]

John D Bogden, Francis W Kemp, Shenggao Han, Wenjie Li, Kay Bruening, Thomas Denny, James M Oleske, Joan Lloyd, Herman Baker, George Perez, Patricia Kloser, Joan Skurnick, and Donald B Louria

ABSTRACT

Background: Immune function is highly dependent on nutritional status because the large mass and high rate of cellular turnover of the immune system make it a major user of nutrients. Furthermore, nutrient requirements may be increased during acute and chronic infections, including HIV-1 infection.

Objective: The current study was designed to assess relations among HIV-1 progression and 11 nutritional and demographic variables.

Design: The participants were 106 HIV-infected outpatients and 29 uninfected control subjects (n = 89 men and 46 women; age range: 35–57 y). The HIV-infected subjects represented a broad range of disease progression.

Results: We found lower concentrations of plasma and erythrocyte magnesium and of erythrocyte reduced glutathione beginning early in the course of HIV-1 infection. Significantly decreased hematocrit and increased serum copper concentration developed only late in the course of the disease. Statistically significant univariate associations were found between the $CD4^+$ T lymphocyte count and hematocrit, plasma magnesium concentration, and plasma zinc concentration. The lowest erythrocyte magnesium concentrations occurred in HIV-infected subjects who consumed alcoholic beverages. Independent variables that were significant joint predictors of $CD4^+$ cell count in multiple regression analyses were hematocrit and plasma free choline and zinc concentrations. These 3 factors together explained 43% of the variability in $CD4^+$ cell counts.

Conclusion: The results provide evidence that compromised nutritional and antioxidant status begin early in the course of HIV-1 infection and may contribute to disease progression. *Am J Clin Nutr* 2000;72:809–15.

KEY WORDS HIV-1 infection, HIV infection, AIDS, HIV progression, glutathione, magnesium, hematocrit, choline, copper, zinc, ethanol, alcohol, antioxidants

INTRODUCTION

It is well known that nutrition has a profound influence on the immune system. Infections, no matter how mild, have adverse effects on nutritional status. Conversely, almost any nutrient deficiency, especially if sufficiently severe, will impair resistance to infection (1). Furthermore, because the immune system functions as a large organ, its size and high rate of cellular turnover make it a major user of nutrients (2). Thus, it is not surprising that nutritional status can greatly influence the course of an acute or chronic infectious disease, especially a severe, chronic infection such as HIV-1. Causes of compromised nutrition in HIV-infected individuals, even in the early stages of HIV infection, may include anorexia, changes in nutrient absorption, and a high level of immune system activity that depletes nutrients (2, 3).

In the current study, we investigated relations between 9 nutritional or biochemical variables and the progression of HIV-1 infection. The variables were hematocrit, erythrocyte concentrations of magnesium and reduced glutathione (GSH), ethanol consumption, and plasma concentrations of magnesium, copper, zinc, and free and total choline. These variables were chosen because each may independently influence the course of an infectious disease or may be altered by an infection, but few studies have assessed their relation to HIV-1 infection and its progression (1, 3–6). The objective of the present study was to assess relations between HIV progression and these variables, both individually and jointly in multiple regression models.

This study was based on 2 hypotheses: *1*) the status of some nutrients, as assessed on the basis of circulating concentrations, will decline early in the course of HIV-1 infection but will not decline further with disease progression, and 2) the status of other nutrients will change progressively with increasing severity of HIV-1 infection or will only be altered in the late stages of infection.

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TABLE 1
Subject characteristics[1]

	BMI[2]	$CD4^+$ cell count
	kg/m²	*10^6 cells/L*
HIV status and sex		
Uninfected		
Female (n = 19)	26.8 ± 2.6	—
Male (n = 10)	26.3 ± 1.1	—
HIV infected		
Female (n = 27)	24.7 ± 1.3	251 ± 47 (7–658)
Male (n = 79)	24.7 ± 0.5	382 ± 30 (1–1129)
HIV stage[3]		
CDC stage C (n = 26)	24.0 ± 0.7	108 ± 18 (1–304)
CDC stage B (n = 44)	24.3 ± 0.8	310 ± 23 (6–628)
CDC stage A (n = 34)	25.9 ± 1.2	600 ± 43 (88–1129)
Uninfected (n = 29)	26.6 ± 1.7	—

[1] $\bar{x}$ ± SE; range in parentheses. Age range: 35–57 y. CDC, Centers for Disease Control and Prevention.

[2] BMI did not differ significantly with infection status or stage (ANOVA, $P > 0.05$).

[3] Stage was unknown for 2 HIV-infected subjects because of insufficient clinical data. Percentage female by stage: C, 46%; B, 14%; A, 24%; uninfected, 66%.

SUBJECTS AND METHODS

Subjects

The subjects in this cross-sectional study were men and women with HIV-1 infection who were outpatients at either of 2 infectious disease clinics at 2 large hospitals in Newark, NJ. Control subjects of comparable ages were recruited from uninfected friends or relatives of the subjects (n = 12) and hospital and medical school employees (n = 17). A total of 106 HIV-infected and 29 uninfected subjects were enrolled. Subjects responded to a 24-item questionnaire that assessed nutritional and health status and included questions about variables such as height and weight; use of prescription medications; consumption of alcoholic beverages; use of vitamin, mineral, or herbal supplements; and symptoms such as anorexia, fatigue, diarrhea, vomiting, and weight loss during the past year. Informed consent was obtained from all subjects and the study protocol was approved by the New Jersey Medical School Institutional Review Board.

HIV-infected subjects were classified into Centers for Disease Control and Prevention (CDC) stages A, B, or C on the basis of the presence of opportunistic infections or other conditions (7), without regard for $CD4^+$ cell counts. The recruitment goal was ≥25 subjects in each category and ≥25 uninfected control subjects. Further subdivision of subjects by both CDC stage and $CD4^+$ cell count was not done because this would have resulted in small numbers of subjects in some subgroups, precluding statistical analysis of the laboratory data.

Blood collection and analysis

We collected 15 mL whole blood on one occasion from each participant. Blood samples were delivered to the laboratory within 4 h of collection. Blood for choline and GSH measurements was collected in evacuated tubes (Becton Dickinson & Co, Rutherford, NJ) containing EDTA as the anticoagulant. Blood used for analyses of magnesium, copper, and zinc concentrations was collected into heparin-treated evacuated tubes recommended for trace metal analysis (Becton Dickinson & Co). Hematocrit was determined on the same sample by using a microhematocrit centrifuge. $CD4^+$ T lymphocyte counts of infected subjects were assessed by using fluorescence-activated cell sorting. Serologic status for HIV infection was confirmed by Western blot. Plasma free and total choline concentrations were measured by using a microbiological method as described previously (8). Plasma copper, zinc, and magnesium and erythrocyte magnesium concentrations were measured by using flame atomic absorption spectrophotometry (9, 10). Erythrocyte concentrations of GSH were measured by visible spectrophotometry at 412 nm with the method of Beutler et al (11).

Statistical analyses

The data were analyzed by using the SAS SYSTEM FOR WINDOWS (release 6.12; SAS Institute, Cary, NC) and are presented as means ± SE. Mean concentrations of biochemical variables and other data for stage-of-infection groups (uninfected and disease stages A, B, and C) were compared by using a general linear models approach to analysis of variance (ANOVA). Groups were compared pairwise by using Tukey's studentized range test when the ANOVA indicated a significant overall group effect. Univariate associations between nutrient concentrations and $CD4^+$ T lymphocyte counts were evaluated by calculating Pearson's product-moment correlation coefficients. Multiple regression analyses with forward selection across disease stages A, B, and C were used to determine relations of $CD4^+$ cell counts to the independent variables hematocrit, erythrocyte magnesium and GSH concentrations, ethanol consumption, age, sex, and plasma magnesium, copper, zinc, and free and total choline concentrations. Stepwise and backward-elimination regressions were also conducted to check the consistency of the results from forward selection. Values for male and female subjects were compared by using t tests. The Mantel-Haenszel chi-square test for trend was used to relate the fractions of subjects with low zinc concentrations to increasing disease severity (uninfected through stage C). All P values for the above tests are two-tailed with $P < 0.05$ considered statistically significant.

For infected participants, we also determined the effects on the 8 biochemical variables of subject-reported changes in appetite or weight, use of vitamin supplements or nutritional drinks, use of antiretroviral medications, and presence of vomiting or diarrhea. These effects were evaluated in pairwise comparisons with t tests. Because 56 comparisons were made, $P < 0.02$ was considered statistically significant for these comparisons; this was done so that the expected number of type I errors would be ≈1.

RESULTS

Subject characteristics

Subject characteristics, including infection status and stage, age, sex, $CD4^+$ cell count, and body mass index (BMI, in kg/m^2), are shown in **Table 1**. We did not have enough information to classify 2 of the 106 infected subjects into CDC categories, but data from these subjects were used for calculations not involving CDC classification. The range of $CD4^+$ cell counts was broad, from 1 to 1129 × 10^6 cells/L. Infected male and female subjects had similar mean BMIs (24.7 ± 0.5 and 24.7 ± 1.3, respectively). BMI did not differ significantly with infection status or stage (ANOVA).

TABLE 2
Biochemical variables by stage of HIV-1 infection[1]

	Uninfected control subjects (n = 28–29)	CDC stage of infection A (n = 29–34)	B (n = 32–44)	C (n = 24–26)	P[2]
Hematocrit	0.419 ± 0.010[a]	0.437 ± 0.008[a]	0.411 ± 0.009[a]	0.375 ± 0.009[b]	0.0003
Erythrocyte magnesium (mmol/L)	2.04 ± 0.05[a]	1.85 ± 0.05[b]	1.90 ± 0.03[a,b]	1.97 ± 0.05[a,b]	0.014
Erythrocyte glutathione (mmol/L)	2.21 ± 0.07[a]	1.54 ± 0.08[b]	1.81 ± 0.10[b]	1.62 ± 0.13[b]	0.0001
Plasma magnesium (mmol/L)[3]	0.82 ± 0.01[a]	0.79 ± 0.01[a,b]	0.77 ± 0.01[b]	0.77 ± 0.01[a,b]	0.0091
Plasma copper (μmol/L)	17.9 ± 0.8[a]	19.5 ± 0.8[a]	20.0 ± 0.6[a]	22.2 ± 0.8[b]	0.0029
Plasma zinc (μmol/L)	13.1 ± 0.4	13.1 ± 0.5	12.6 ± 0.5	11.9 ± 0.6	NS
Plasma free choline (μmol/L)	43.6 ± 2.3	51.3 ± 3.2	74.3 ± 18.2	48.0 ± 3.6	NS
Plasma total choline (mg/L)	293 ± 8	306 ± 11	316 ± 11	301 ± 12	NS

[1] $\bar{x}$ ± SE. Values in the same row with different superscript letters are significantly different, $P < 0.05$ (Tukey's test). Tukey's test was done when $P < 0.05$ for ANOVA. CDC, Centers for Disease Control and Prevention.
[2] ANOVA for effect of serologic status and stage of HIV-1 infection.
[3] For the pairwise comparison between uninfected control and stage-C subjects, $0.05 < P < 0.062$.

Nutrient concentrations and stage of infection

Five of the 8 biochemical variables studied differed significantly with stage of infection (ANOVA, $P < 0.05$; **Table 2**). Hematocrit values were significantly lower and plasma copper concentrations were significantly higher in stage-C subjects than in the other 3 groups. For plasma magnesium concentrations, we found significant differences between uninfected subjects and stage-B but not stage-A subjects. A comparable difference between plasma magnesium concentrations in uninfected and stage-C subjects was not statistically significant ($0.05 < P < 0.062$) because fewer subjects were classified as stage C than as stage B. Erythrocyte magnesium concentrations were significantly lower in stage-A but not in stage-B or -C subjects than in control subjects. Stage of infection did not influence mean plasma zinc, free choline, or total choline concentrations significantly. However, the lowest zinc concentrations occurred in stage-C subjects: 36% had concentrations below the normal range of 10.7–18.3 μmol/L (70–120 μg/dL). The percentages of uninfected and stage-A and -B subjects with values <10.7 μmol/L were 14.3%, 14.7%, and 30.2%, respectively. These percentages show a significant increase in below-normal zinc concentrations with infection and increasing disease severity (Mantel-Haenszel chi-square test for trend, $P < 0.05$).

At all 3 stages of HIV infection, erythrocyte GSH concentrations were significantly and considerably lower than those of uninfected control subjects. Individual erythrocyte GSH values of uninfected subjects and infected subjects at each CDC stage are shown in **Figure 1**. A considerable percentage (37.2%) of the infected subjects had concentrations lower than the lowest value of any uninfected control subject.

Laboratory data by subject sex

Because the percentages of male and female study participants (Table 1) differed among the 4 groups of subjects (uninfected and stages A, B, and C), we compared laboratory data of the male and female subjects for the 8 biochemical variables listed in Table 2. For the 29 uninfected subjects, there was a significant difference between female and male subjects for hematocrit (0.397 ± 0.013 compared with 0.481 ± 0.005, respectively) and plasma copper concentration (18.9 ± 1.1 compared with 15.9 ± 0.6 μmol/L, respectively) by t test ($P < 0.05$). For the 106 HIV-infected subjects, men had significantly higher values than women for hematocrit (0.426 ± 0.006 compared with 0.369 ± 0.008, respectively) and plasma zinc concentration (13.0 ± 0.4 compared with 11.6 ± 0.5 μmol/L, respectively). For subjects with stage-C infection, only plasma zinc concentration differed between female and male subjects (10.1 ± 0.7 compared with 13.6 ± 0.8 μmol/L, respectively). The only variable that differed between men and women at stages A and B was hematocrit. For stage A, the mean values were 0.453 ± 0.007 and 0.386 ± 0.018 for men and women, respectively; for stage B, the means were 0.423 ± 0.009 and 0.335 ± 0.010 for men and women, respectively. There were no other significant differences in the measured variables between men and women. Erythrocyte GSH concentrations were remarkably similar in women and men for uninfected subjects and those at each stage of infection. For example, mean concentrations were 2.22 ± 0.10 and 2.18 ± 0.10 mmol/L for uninfected female and male subjects, respectively, and 1.53 ± 0.19 and 1.69 ± 0.18 mmol/L for stage-C female and male subjects, respectively.

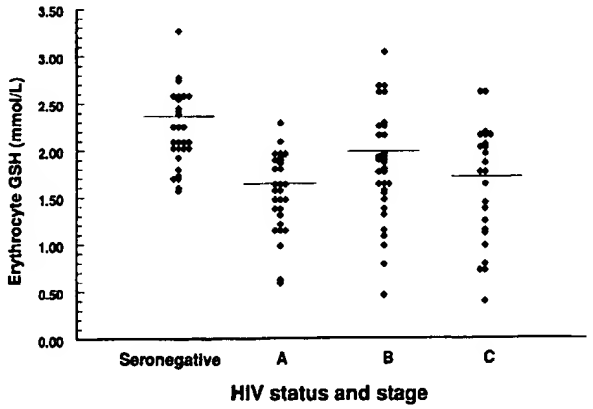

FIGURE 1. Erythrocyte reduced glutathione (GSH) concentrations by HIV status and Centers for Disease Control and Prevention stage for individual subjects. Mean values are indicated by horizontal lines and differ significantly (ANOVA, $P < 0.0001$). The percentage of infected subjects with GSH concentrations below the lowest value for any uninfected control subject was 37.2%. n = 29 for seronegative, 29 for stage A, 32 for stage B, and 24 for stage C.

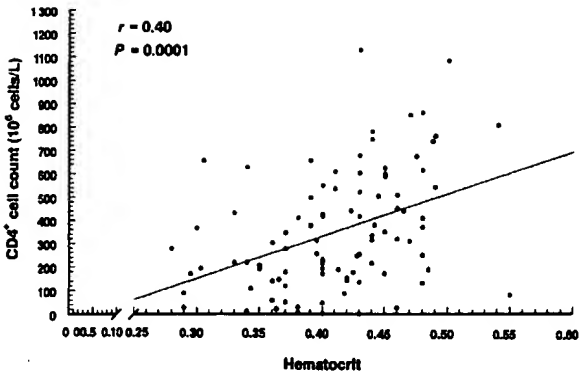

FIGURE 2. Relation of CD4⁺ cell count to hematocrit (n = 94). The relation between the variables is best described by a straight line defined by the equation $y = ax + b$, where y = CD4⁺ cell count, x = hematocrit, a = 1800, and b = −388.

Alcoholic beverages

Mean ethanol consumption of those subjects who drank alcoholic beverages was 4.6 ± 3.5 g/d (≤2.6% of energy) for uninfected subjects and 9.9 ± 2.0 g/d (≤4.4% of energy) for infected subjects. No stage-C subjects reported consumption of alcoholic beverages. However, ethanol consumption did not differ significantly by infection status or stage of infection.

Each subject was classified into 1 of 4 groups on the basis of infection status (uninfected or infected) and use of alcoholic beverages (did or did not consume alcohol). For subjects who did not consume alcoholic beverages, mean erythrocyte magnesium concentrations were 2.03 ± 0.09 mmol/L for 7 uninfected subjects and 1.91 ± 0.04 mmol/L for 46 infected subjects. Corresponding values for uninfected (n = 5) and infected (n = 16) subjects who drank alcoholic beverages were 2.02 ± 0.12 and 1.76 ± 0.05 mmol/L, respectively. The infected subjects who consumed alcoholic beverages had significantly lower erythrocyte magnesium concentrations than did subjects in the other 3 groups (ANOVA, $P < 0.05$).

Relations between nutrient concentrations and CD4⁺ T lymphocyte counts

In **Figures 2, 3,** and **4** we show the relations between CD4⁺ T lymphocyte count and hematocrit, plasma magnesium concentration, and plasma zinc concentration, respectively. These were the only variables that were significantly ($P < 0.05$) associated with CD4⁺ cell counts. Linear correlation coefficients were 0.40 for hematocrit, 0.25 for plasma magnesium concentration, and 0.21 for plasma zinc concentration. The association between CD4⁺ cell count and plasma free choline concentration was not significant (r = 0.20, P = 0.077). Additionally, there were no significant associations between plasma or erythrocyte magnesium concentrations and erythrocyte GSH concentration. Erythrocyte magnesium and GSH concentrations were not significantly associated with hematocrit.

The results of the multiple regression analyses performed by using forward selection with CD4⁺ cell count as the dependent variable are shown in **Table 3**. Similar results were obtained by using a stepwise approach or backward elimination (data not reported). The independent variables were hematocrit, erythrocyte magnesium and GSH concentrations, ethanol consumption, age, sex, and plasma magnesium, copper, zinc, free choline, and total choline concentrations. Independent variables that were significant joint predictors of CD4⁺ cell count were hematocrit (partial R^2 to enter model = 0.27), plasma free choline concentration (R^2 = 0.087), and plasma zinc concentration (R^2 = 0.067). Together, these 3 variables explained 43% of the variability in CD4⁺ cell counts. The absence of plasma magnesium concentration in the multiple regression model appears to be a result of its association with hematocrit (r = 0.19, P = 0.027).

Symptoms, supplements, and medications

Responses to questions about nutrition and medication use during the past year were tabulated. Of the 106 infected subjects, 24 (22.6%) reported reduced appetite, 17 (16.0%) reported moderate weight loss, 12 (11.3%) reported episodes of vomiting, and 21 (19.8%) reported diarrhea. Fifty-six subjects (52.8%) were taking antiretroviral drugs at the time of blood sampling and 25 (23.6%) reported a recent weight loss of ≥2.25 kg (5 lb). Fifty subjects (47.2%) reported use of vitamin supplements and 38 (35.8%) consumed nutritional drinks. Of these drinks, the most frequently used was Sustacal (n = 28). The above percentages were highest for the 26 stage-C subjects, with the exception of the percentage reporting diarrhea.

Weight loss of ≥2.25 kg, use of antiretroviral drugs, diarrhea, and use of vitamin or herbal supplements did not significantly ($P < 0.02$) influence any of the 8 biochemical variables measured. Hematocrit was significantly lower ($P < 0.02$) in subjects who reported loss of appetite (0.381 ± 0.013 compared with 0.418 ± 0.006) or use of nutritional drinks (0.390 ± 0.010 compared with 0.421 ± 0.007) than in subjects who did not. Significantly lower plasma zinc concentrations were found in subjects who reported loss of appetite (11.0 ± 0.6 compared with 13.1 ± 0.4 μmol/L) or vomiting (10.6 ± 0.5 compared with 12.9 ± 0.4 μmol/L) than in subjects who did not. No other significant differences were found for subjects who reported loss of appetite, vomiting, or use of nutritional drinks.

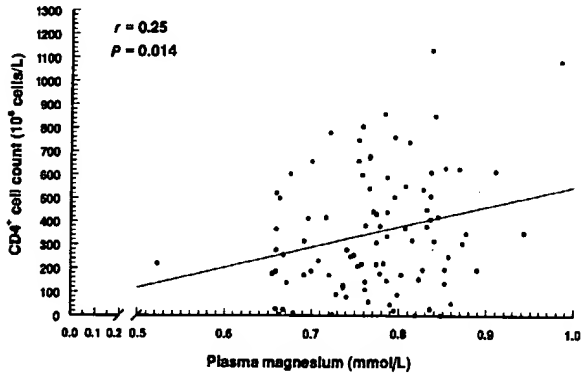

FIGURE 3. Relation of CD4⁺ cell count to plasma magnesium concentration (n = 94). The relation between the variables is best described by a straight line defined by the equation $y = ax + b$, where y = CD4⁺ cell count, x = plasma magnesium concentration, a = 862, and b = −314.

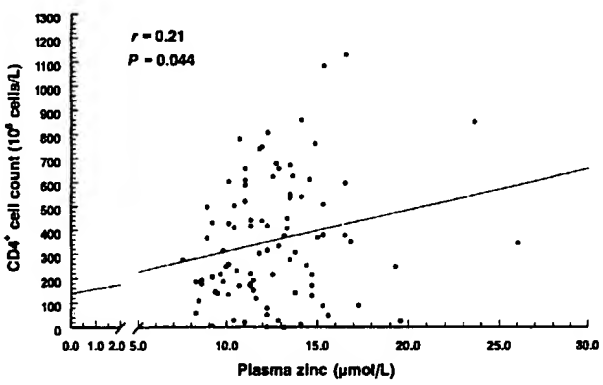

FIGURE 4. Relation of CD4⁺ cell count to plasma zinc concentration (n = 92). The relation between the variables is best described by a straight line defined by the equation $y = ax + b$, where y = CD4⁺ cell count, x = plasma zinc concentration, a = 17.2, and b = 141.

DISCUSSION

The HIV-infected subjects who participated in this study were classified into CDC stages A, B, or C on the basis of their symptoms and the presence of opportunistic infections. The mean (±SE) CD4⁺ cell counts decreased substantially with increasing severity of infection (600 ± 43, 310 ± 23, and 108 ± 18 × 10^6 cells/L for stages A, B, and C, respectively) and thus were consistent with the CDC classification of the infected subjects. Nevertheless, CD4⁺ cell counts and stage of infection provide different measures of disease progression, and this must be considered when interpreting the results of this study.

Differences between uninfected and infected subjects were particularly large for erythrocyte GSH; concentrations were already low early in the course of HIV infection, but there was considerable variability among infected subjects, especially those in stages B and C (Figure 1). However, erythrocyte GSH concentrations were not lower with increasing severity of HIV infection. Thus, GSH was not associated with disease progression as assessed by CD4⁺ cell counts or stage of disease of infected subjects. Nevertheless, because GSH is a key cellular antioxidant, the relatively low concentrations found even early in the course of infection may contribute to HIV pathogenesis, especially because other investigations have found evidence that compromised status of antioxidants is associated with HIV progression. For example, Herzenberg et al (12) found that CD4⁺ lymphocyte glutathione concentrations have prognostic value for predicting the rate of progression when measured in asymptomatic seropositive patients. These authors suggested that the use of acetaminophen and other drugs known to deplete GSH should be minimized or avoided in persons with HIV infection.

Garcia de la Asuncion et al (13) found that mitochondrial glutathione oxidation increases substantially with aging in rats and mice, with a remarkably high degree of correlation between oxidized glutathione and mitochondrial DNA damage (r = 0.95–0.98). The present study focused on GSH concentrations in erythrocytes. However, if the decreased GSH concentrations that we found at all stages of infection are associated with mitochondrial DNA damage, then the latter could be an important adverse effect of HIV-1 infection.

Subjects who were infected and also drank alcoholic beverages had the lowest erythrocyte magnesium concentrations. This is not surprising because seropositive status and consumption of ethanol are each associated with decreased circulating magnesium concentrations (9, 14). The ethanol intake of infected subjects who consumed alcoholic beverages was low ($\bar{x}$: ≤4.4% of energy). The fact that no stage-C subjects reported regular consumption of alcoholic beverages may have contributed to their higher erythrocyte magnesium concentrations compared with those of the stage-A and -B participants. These data suggest that HIV-infected subjects who consume alcoholic beverages, even in modest amounts, may be especially likely to develop compromised magnesium status.

Relatively high plasma copper concentrations, which we found in the stage-C subjects, were also observed by other investigators and most likely reflect a nonspecific increase in plasma concentrations of the copper-containing protein ceruloplasmin (15, 16). Plasma concentrations of ceruloplasmin and copper increase as an acute-phase response in a variety of infections and inflammatory conditions, and thus are not specific for HIV infection. We did not observe significantly lower plasma zinc concentrations with disease progression (as assessed by stage of infection) in HIV-positive subjects as was found in some other studies (17, 18), although the stage-C subjects did have the lowest mean concentration (11.9 μmol/L) and the highest percentage of subjects with below-normal values (36%). In addition, there was a significant association between plasma zinc concentration and CD4⁺ cell count. These observations are consistent with the idea that CD4⁺ cell count and CDC stage provide different, although complementary, measures of disease progression.

Hematocrit values were lowest in the stage-C subjects. This result agrees with other studies in which reduced hematocrit values were found late in the course of HIV infection (19–22). In addition, the development of anemia is an independent risk factor for an early death in HIV-infected individuals (19–22). Several studies showed that HIV-infected patients with anemia may benefit from treatment with recombinant human erythropoietin (23, 24), which can improve survival. In our multiple regression model, hematocrit was the independent variable that was the best predictor of CD4⁺ cell count. In addition, plasma zinc and free choline concentrations were significant joint predictors of the CD4⁺ cell count. There is considerable evidence that zinc is vital to cellular immune function. Choline was recognized as an essential dietary nutrient by the Institute of Nutrition of the National Academy of Sciences, and dietary reference intakes have been established for it (25). Because choline is required for cellular functions such as phospholipid synthesis (25), it is

TABLE 3
Multiple regression models of CD4⁺ cell counts on biochemical variables[1]

Predictor	Partial R^2	Parameter estimate	P
Intercept		−1354	0.0020
Hematocrit	0.273	1880	0.0005
Plasma free choline (μmol/L)	0.087	580	0.029
Plasma zinc (μmol/L)	0.067	15.6	0.045
Plasma magnesium (mmol/L)	0.012	469	0.38

[1] None of the other 7 independent variables (erythrocyte magnesium and GSH concentrations, ethanol consumption, age, sex, and plasma copper and total choline concentrations) met the 0.50 signficance level for entry into the model. Forward selection was used to enter independent variables into the model. The model R^2 = 0.439 (n = 42).

plausible that serum concentrations might be depleted with progression of HIV infection as the immune system attempts to respond to progression with increased synthesis of cells and molecules such as cytokines. Although $CD4^+$ cell counts were significantly associated with free choline concentrations in the multiple regression models, there were no significant differences in mean choline concentrations between the different stages of infection. This is an example of $CD4^+$ cell counts and stage of infection having different relations to the status of a nutrient.

In Figures 3, 4, and 5, we illustrated the significant associations between $CD4^+$ cell count and hematocrit, plasma magnesium concentration, and plasma zinc concentration, respectively. Despite significant group associations, in these figures it is evident that some individual points were situated a substantial distance from the regression line. Therefore, an individual's $CD4^+$ cell count cannot be predicted from laboratory data on hematocrit or plasma concentrations of magnesium or zinc. This is not surprising because numerous factors contribute to the $CD4^+$ cell counts of individual HIV patients and any single nutritional variable is likely to have only a moderate association with $CD4^+$ cell count. However, the 4 nutritional variables in the multiple regression model collectively explained ≈43% of the variability in $CD4^+$ cell counts. Because hematocrit is frequently measured in clinical medicine, it may be particularly useful for monitoring individual HIV-infected patients.

Limitations of the present study are that the results describe associations that may not represent a causal relation and that blood concentrations are only one measure of nutrient status.

Furthermore, it is unlikely that compromised nutritional status functions in isolation to influence the progression of HIV-1 infection, but rather that it acts in concert with other factors such as viral load and genetics. Compromised nutritional status that develops early in the course of infection, for example, the considerable changes in GSH status that we found, may exert its most substantial adverse effects only after interacting with other factors that result in further deterioration of host defenses. It could be argued that treatment of HIV-1 infection with drugs may contribute to compromised nutrient status because of drug side effects such as anorexia and diarrhea. Alternatively, antiretroviral therapy may improve nutritional status if anorexia is present because of infection-produced effects on cytokines. However, in the current study, use of antiretroviral drugs, anorexia, and diarrhea, as well as subject sex, had little or no effect on the status of the nutrients studied. Thus, the effects of these nutrients on HIV progression may be independent of the above factors.

The present study focused on assessing relations between HIV progression and magnesium, copper, zinc, choline, and glutathione. Other studies suggested that other micronutrients, for example, vitamins A, B-6, and B-12 and selenium (26–29), may also influence HIV-1 progression. Thus, the results of this study add to the body of evidence showing compromised antioxidant, mineral, and micronutrient nutritional status during HIV infection. For some nutrients, this occurs early in the course of HIV infection. Because the major minerals and micronutrients play key roles in supporting immune function, compromised status of these nutrients may contribute to the progression of HIV-1 infection.

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Contribution of zinc to reduce $CD4^+$ risk factor for 'severe' infection relapse in aging: parallelism with HIV

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Abstract

Aging and HIV have parallelism in immunodeficiency status because of the appearance of infections or relapse leading to death in both conditions. HIV-RNA is predictor for HIV progression correlated with $CD4^+$ depletion. $CD4^+$ and plasma zinc levels (zincaemia) may be predictors for infections relapse in aging because of zinc relevance for normal immune efficieny against infections and for $CD4^+$ growth. Moreover, zincaemia decreases in aging and infection. A total of 67 elderly subjects affected by infections resistant to antibiotic therapy were recruited. A total of 28 HIV^+ subjects with HAART therapy were also used. $CD4^+$ depletion (507 mm^3) and zincaemia deficiency (76 μg/dl), as compared to $CD4^+$ (700–1100 mm^3) and zincaemia (85–100 μg/dl; age 40–75 years) normal ranges, are possible limits (Cox hazard regression) for severe infections relapse, such as chronic obstructive bronchitis and bronchopneumonia by bacteria or Candida complication, in aging. $CD4^+$ and zincaemia values are within the lower limits of normal range in urinary tract infections. Zincaemia and HIV-RNA or $CD4^+$ are inversely correlated ($r = 0.57$ and $r = 0.72$, respectively) in HIV^+ HAART treated subjects. Consequently there is no appearance of opportunistic infections. Parallelism between aging and HIV may exist because of the resemblance in marked zinc deficiency and $CD4^+$ depletion with high scores in relative risks for severe infections relapse. Supplementing zinc (12 mg Zn^{++}/day) for one month in infected elderly subjects and HAART therapy in HIV^+ subjects reduces risk scores in $CD4^+$ and zincaemia deficiencies for infections relapse, suggesting that the zinc beneficial effect may be independent

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either by HIV-virus or pathogen agents involved. While HAART may reduce the occurrence of opportunistic infections in HIV by means of also major zinc bioavailability, supplementing zinc can be recommended in elderly people as resistence to infections. Since zinc deficiecy is correlated with $CD4^+$ depletion, this latter may also be good diagnostic marker to detect 'clear immunodeficency' in aging, as in HIV condition.

Keywords: Aging; HIV; Zinc; $CD4^+$; HIV-RNA; Infection relapse; Risk factors

1. Introduction

Aging and HIV have parallelism in immunodeficiency status because of the appearance of infections or relapse leading to death in both conditions (Pawelec & Solana, 1997). Proteases inhibitors reduce opportunistc infections (Chaisson & Moore, 1997) because of HIV-RNA relevance for HIV progression as major predictor (Mellors, Rinaldo, Gupta, White, Todd & Kingsley, 1996), other than $CD4^+$ depletion (Levy, 1989). $CD4^+$ count dysregulation causes infections or infections recidive (Pawelec & Solana, 1997). No specific immunological limits under which risk of infection or infection relapse are still available in aging. Together with $CD4^+$, zinc may be a predictor because: (i) zinc is important for immune efficiency against infections (Sugarman, 1983); (ii) zinc is predictor for death in comatose patients for high frequency of infectious episodes (Mocchegiani, Imberti, Testasecca, Zandri, Santarelli & Fabris, 1995a); (iii) zinc deficiency is a cofactor for infections in old age (Chandra, 1989). Finally zinc affects $CD4^+$ maturation and differentiation (Beck, Prasad, Kaplan, Fitzgerald, & Brewer, 1997). Moreover zinc decreases in aging (Fabris, Mocchegiani, Amadio, Zannotti, Licrasto & Franceschi, 1984) and during infections (Mocchegiani Veccia, Ancarani, Scalise, & Fabris, 1995b; Sandstead, 1994; Shankar & Prasad, 1998), whereas $CD4^+$ depletion occurs during infections (Pawelec & Solana, 1997). In turn, infections and infections relapse are constant events in aging (Fabris & Mocchegiani, 1995). Plasma zinc levels (zincaemia) and $CD4^+$ are tested in infected elderly subjects from remission phase as possible limits and, as such, possible risk factors for infections relapse resistant to conventional antibiotic therapy (Lewis & Reeves, 1994). Peripheral T-cells are used to give more complete picture of generalized immunodeficiency in aging, as in canine bronchoalveolar infections (Dirscherl, Beisker, Kremmer, Mihalkov, Voss & Ziesenis, 1995). HIV model with HAART therapy was used to further study the possible parallelism between HIV and aging for infections relapse incidence. Because of zincaemia heterogeneity in aging (Fabris et al., 1984; Prasad, Fitzgerald, Hess, Kaplan, Pelen & Dardenne, 1993) and HIV (Fabris, Mocchegiani, Galli, Irato, Lazzarin & Moroni, 1988; Mocchegiani et al., 1995b), total thymulin (TT) (active zinc-bound ZnFTS + inactive zinc-unbound FTS) and active thymulin (ZnFTS) (AT) ratio, which is the unsaturated fraction of thymulin (FTS) by zinc ions (Fabris et al., 1984) is a good marker to detect real zinc deficiency because of the inverse strict correlation between zincaemia status and the ratio itself (Fabris et al., 1984). Thus, zincaemia was associated with TT/AT ratio. Supplementing zinc from remission phase was carried out in elderly subjects affected by pre-existing chronic

obstructive bronchitis. Relative risk factors were calculated from the remission phase (time 0) of infection to month 4 of observation. An HIV model was used as comparison.

2. Subjects and methods

2.1. Elderly subjects

A total of 48 male and 19 female subjects (63–75 years, mean 69 ± 5.8) admitted to the hospital were recruited in remission phase (time 0) of infection (urinary tract, chronic obstructive bronchitis and bronchopneumonia by bacteria or candida complication). Old patients are divided according to infections. Group A with urinary tract infections (18 subjects, 5 male and 13 female, mean age 70 ± 5 years), group B with chronic obstructive bronchitis (36 subjects, 25 male and 11 female, mean age 67 ± 4 years) and group C with bronchopneumonia by bacteria or candida complication (13 subjects, 10 male and 3 female, mean age 68 ± 3.5 years). Elderly patients with previous infections associated with other pathological conditions were excluded. Conventional antibiotic therapy was used before remission phase. Length of conventional antibiotic treatment was of 12 ± 2.0 and 14 ± 2.7 days before time 0 (Data are the mean number of days of antibiotic treatment) for group A and for groups B and C, respectively, with conventional therapeutic dose dependent by infection severity. No other drugs were administered other than Vitamins B complex in order to maintain a good balance of vitamins B by intestinal bacterial flora, which can be destroyed by antibiotic therapy. Blood withdrawal (time 0) was perfomed in remission phase of infection. The daily diet was similar to healthy old controls. Double-blinded controlled trial with zinc at the dose of 12 mg Zn^{++}/day (USDA, 1976) or placebo (starch containing capsule) was carried out for one month in 15 (13 male and 2 female, mean age 68 ± 3 years) and 14 (11 male and 3 female, mean age 66 ± 2 years) elderly people subjects, respectively, after one week from remission phase of infection (chronic obstructive bronchitis). Relative risk factors as well as possible infection relapse were evaluated from month 0 to 4 of observation. Supplementing zinc was carried out in chronic obstructive bronchitis because of more common infection relapse in aging (Pawelec & Solana, 1997) with consequent major subjects availability.

2.2. HIV^+ subjects

A total of 21 male and 7 female intravenous drug-users HIV^+ subjects (20–36 years, mean 28 ± 5.5) were recruited with $CD4^+$ number between 250 and 400 mm^3 (Stage III of disease) (Murray et al., 1985). Because of HIV-RNA as predictor for HIV progression (Mellors et al., 1996) with HAART to block virus replication (Sepkowitz, 1998), the medical ethic suggested to immediatly perform HAART when $CD4^+ \leqslant 400$–500 mm^3 and HIV-RNA $\geqslant 10{,}000$ copies/ml (Sepkowitz, 1998). No other drugs administered other than HAART [2 nucleoside analogues (AZT + 3TC) and 1 proteases inhibitor (IND or RIT)] at standard doses/day (Sepkowitz, 1998), with compliance of 92%. HIV-RNA, immunological and nutritional parameters were detected at months 0 and 4 after HAART therapy as well as possible opportunistic infections censoring. Relative risk factors were evaluated from month 0 to 4 of observation. The data of

relative risk factors were compared with HIV^+ subjects (18 subjects) with $CD4^+$ between 250 and 400 mm^3 treated with only AZT because of no availability of other antiviral drugs in this retrospective study (Mocchegiani et al., 1995b). The daily diet was similar to young healthy controls. No vegetarian subjects were present. ELISA and Western blot confirmed HIV seropositivity.

2.3. Control groups

Seven men and eight women healthy young subjects (25 ± 3.4 years) admitted to the hospital for minor surgery served as controls. Five men and ten women healthy elderly subjects (67 ± 4.6 years) were recruited in accordance to immunogerontological 'SENIEUR protocol' (Ligthart et al., 1984). Vegetarian subjects were excluded. They were recruited and examined according to the guidelines of Helsinki declaration. The informed consensus for blood withdrawls as well as for zinc trial was obtained.

2.4. Methods

HIV-RNA plasma detection was performed by means of RT-PCR amplification of specific primers (SK38, SK39) (Menzo, Bagnarelli, Giacca, Manzin, Varaldo & Clementi, 1992). TT/AT ratio were tested in the plasma by inhibiting azathioprine-sensitive rosettes assay (Fabris et al., 1984). Zincaemia was tested using A.A.S. (Fabris et al., 1984). $CD4^+$ absolute number was counted with cytofluorimetry (Epics V, Culter, USA) using Leu3a FITC (Becton-Dickinson, USA). Differences were compared using ANOVA (two-way). Cox hazard regression (Matthews & Farewell, 1988) was used to test relative predictors ($CD4^+$ and zincaemia) for infection risks and for possible limits under which rinfections risk may be high. Least-square method and analysis of covariance were used for correlations and to test significance among regression lines, respectively. Differences were significant when $P \leqslant 0.05$.

Table 1
Immunological and nutritional parameters in elderly subjects in remission phase of infection (time 0)[a]

Groups[b]	Ratio T.T./A.T. (log_{-2})	Zincaemia (μg/dl)	CD 4^+ (mm^3)	No of infections/subjects
A	2.0 ± 0.3	83.3 ± 4.0	696 ± 58	18/18
B	$2.5 \pm 0.3^*$	$75.3 \pm 3.2^{**}$	$457 \pm 53^*$	13/13
C	$2.4 \pm 0.2^*$	$76.8 \pm 4.3^{**}$	$460 \pm 66^*$	36/36
D	1.6 ± 0.2	88.5 ± 5.8	877 ± 47	
E	1.1 ± 0.2	120.0 ± 6.5	925 ± 68	

[a] $^*P < 0.01$ and $^{**}P < 0.05$ when compared to group D.

[b] A = Elderly subjects ($n = 18$) with pre-existing infections (urinary tract); B = Elderly subjects ($n = 13$) with pre-existing infections (bronchopneumonia by bacteria or Candida complication); C = Elderly subjects ($n = 36$) with pre-existing infections (Chronic obstructive bronchitis); D = Elderly age-matched healthy controls ($n = 15$) (age 60–73 years); E = Young healthy controls ($n = 15$) (age 20–30 years).

3. Results

3.1. Elderly subjects

TT/AT ratio >2 (Groups B and C) is associated with more marked zinc deficiency as compared to TT/AT ratio (=1.6) and zincaemia values of old healthy controls ($P < 0.01$) (Table 1). The anamnestic analysis at time 0 (remission phase) shows that urinary infections (18/18 patients), bronchopneumonia [by bacteria (8/13 patients) or by Candida complication (5/13 patients)] and chronic obstructive bronchitis (36/36 patients) are pre-existing infections. $CD4^+$ count is reduced (groups B and C) ($P < 0.01$), whereas no difference in group A as compared to old healthy controls (Table 1). $CD4^+$ (437 ± 49 mm^3) and zincaemia (74 ± 3.4 μg/dl) values in old patients affected by bronchopneumonia by Candida complication are not different as compared to those ones by bronchopneumonia by bacteria complication ($CD4^+ = 478 \pm 62$ mm^3; zincaemia $= 76 \pm 3.8$ μg/dl). No differences in $CD4^+$ and zincaemia exist between old patients affected by chronic obstructive bronchitis and by bronchopneumonia (Table 1), as well as between male and female (data not shown). $CD4^+$ and zincaemia values for urinary tract infections are included within the normal range (Table 1). Supplementing zinc for one month from remission phase (time 0) in elderly subjects affected by chronic obstructive bronchitis increases $CD4^+$ cell number (from 462 ± 48 time 0 to 690 ± 41 time 1 month; $P < 0.01$) with significant reduction of infections relapses (2 relapses/15 patients = 13.3%) as compared to placebo group (6 relapses/14 patients = 42.8%) ($P < 0.01$) at month 4 of observation. $CD4^+$ depletion and zinc deficiency in placebo group are relative risk factors for infections relapse with significant scores as compared to old zinc-treated group (Table 2). Moreover, taking into account $CD4^+$ and zincaemia normal ranges (700–1100 mm^3; 20–75 years; 85–100 μg/dl; 40–75 years, respectively) from our laboratory, $CD4^+$ (507 mm^3) (range 454–530) and zincaemia (76 μg/dl) (range 75–91) may be possible limits at borderline for chronic obstrucutive bronchitis infections relapse. When the data of zincaemia from old infected patients before and after zinc supplementation and respective healthy controls are plotted against the corresponding values of $CD4^+$, significant positive inverse correlation is found ($r = 0.81$ $P < 0.01$).

Table 2
Relative risk factors for chronic obstructive bronchitis relapse in elderly subjects treated with zinc and for opportunistic infections in HIV^+ subjects treated with AZT (retrospective study) or HAART

Covariates	Coeff. Regr.	Rel. Risk	z-statistic	Coeff. Regr.	Rel. Risk.	z-statistic
	Placebo ($n = 14$)			Zinc treatment ($n = 15$)		
$CD4^+$	0.82	2.27	$P < 0.01$	0.36	1.44	$P > 0.05$
zincaemia	0.71	2.03	$P < 0.01$	0.41	1.51	$P > 0.05$
	HIV^+ (AZT treatment) ($n = 18$)			HIV^+ (HAART treatment) ($n = 28$)		
$CD4^+$	0.78	2.18	$P < 0.01$	0.51	1.67	$P > 0.05$
zincaemia	0.85	2.34	$P < 0.01$	0.48	1.62	$P > 0.05$

3.2. HIV^+ subjects

TT/AT ratio > 2 is associated with more marked zinc deficiency at time 0 as compared to day 120 of observation (Table 3). $CD4^+$ and body weight (Δ%) are reduced at time 0 as compared to young healthy controls ($P < 0.001$) (Tables 3 and 1). HAART increases zincaemia, body weight and $CD4^+$ and reduces HIV-RNA (copies/ml) as compared to time 0 ($P < 0.01$) (Table 3). This reduction is also >1 $\log_{10}$. HAART avoids $CD4^+$ depletion and zinc deficiency as relative risk factors for opportunistic infections, as compared to AZT HIV^+ treated subjects (Table 3). HAART induces no appearance of opportunistic infections during the period of observation as compared to AZT treated group (Stage III of disease) of the restrospective study (0 infections vs 13 opportunistic infections among whom, three by Candida) (Mocchegiani et al., 1995b). No difference between male and female and between drug-users and heterosexsuals is found (data not shown). Significant inverse correlations exist between zincaemia and HIV-RNA ($r = 0.57$; $P < 0.05$), between $CD4^+$ and HIV-RNA ($r = 0.72$, $P < 0.01$) and between zincaemia and body weight ($r = 0.76$, $P < 0.01$) in HAART treated HIV^+ subjects.

4. Discussion

$CD4^+$ depletion and marked zinc deficiency are cross-linked in elderly subjects for infections relapse, such as bronchopneumonia and chronic obstructive bronchitis, resistant to conventional antibiotic therapy. HAART induces no appearance of opportunustic infections because of increased $CD4^+$ and zincaemia values, which are both inversely correlated with HIV-RNA. HAART reduces also $CD4^+$ and zincaemia relative risk factors for opportunistic infection incidence. Supplementing zinc in elderly infected subjects restores $CD4^+$ cell number with significant infections relapse reduction, as it occurs in HIV^+ HAART treated subjects. Thus marked zinc deficiency and $CD4^+$ depletion may be considered as probable predictors for infections relapse in aging with HIV parallelism.

$CD4^+$ number is similar in young and old subjects (Cakman Rohwer, Schutz, Kirchner, & Rink, 1996). FasL-apoptosis by bacteria endotoxins may cause $CD4^+$ dysregulation with consequent increased bacteria infections relapse (Pawlec & Solana, 1997; Castro, Bremer,

Table 3
$CD4^+$, zincaemia and HIV-RNA in HIV^+ subjects treated with HAART from day 0–120 of observation[a]

	Ratio TT/AT ($\log_{-2}$)	Zincaemia (μg/dl)	$CD4^+$ (mm^3)	HIV-RNA (copies/ml)	Body weight[b] (Δ%)
t_0	2.33±0.2	78.4±6.4	350±38	28.15±9.36	−1.77±1.23
t_{120}	0.97±0.2*	95.6±5.5*	575±41**	2.49±1.91*	+0.51±0.27*

[a] *$P < 0.01$ and **$P < 0.05$ when compared to time 0. No opportunistic infections occur in HIV^+ HAART treated subjects during the period of observation.

[b] The body weight was calculated as individual variance (Δ%) of the body weight from day 0–120 for each patient. The significance of Δ% = χ^2 test.

Nobrega, Countinho, & Truffa-Bachi, 1998). Indeed, $CD4^+$ depletion is significant in presence of pre-existing infections (bronchopneumonia and chronic obstructive bronchitis). Such a depletion may be more related to previous infections rather than to conventional antibiotic therapy effect because of the blood withdrawals in remision phase and because of the non-influence of antibiotics (independently by doses used or pathogen agent involved) on altered $CD4^+$ subsets in peripheral blood by adult infected subjects (Kawakami et al., 1997), confirming also, at least, the antibiotic therapy poor efficacy in elderly infected subjects (Lewis & Reeves, 1994). Zincaemia and $CD4^+$ decrease during infections (Pawelec & Solana, 1997; Fabris et al., 1988; Mocchegiani et al., 1995b; Sandstead, 1994), which often are, in turn, age-associated diseases (Fabris & Mocchegiani, 1995). Because of no availability of specific immunological limits under which infection relapse risk may be high in old age, zincaemia and $CD4^+$ may be taken into account because: (i) zinc is important for immune efficiency against infections by virus, fungi and bacteria (Sugarman, 1983); (ii) zinc deficiency is a predictor of death in comatose patients suffering by numerous infectious episodes (Mocchegiani et al., 1995a); (iii) zinc deficiency is a cofactor for infection in aging (Chandra, 1989); (iv) zinc shows an age-related decrease (Fabris et al., 1984) and it is essential for $CD4^+$ maturation and growth (Beck, Prasad, Kaplan, Fitzgerald & Brewer, 1997). Following that, zinc deficiency and $CD4^+$ decrement may be cross-linked with possible limits under which infections relapse risk may be high. Because of no immune differences between chronic obstructive bronchitis and bronchopneumonia, such limits may be the same and both are reduced as compared to $CD4^+$ (Hannet, Erkeller-Yuksel, Lydyard, Deneys, & DeBruyere, 1992) and zincaemia normal ranges (Fabris et al., 1984). $CD4^+$ and zincaemia limits are within the lower limits of normal ranges in urinary tract infections. Thereby, these latter may be considered as 'moderate', whereas the others as 'severe' (Laurence, 1993). Without excluding other T-subsets dysregulations, such as 'memory' and 'naive' T-cells, in aging (Sansoni et al., 1993) and in infections (Pawelec & Solana, 1997), because of significant inverse correlation between $CD4^+$ and zincaemia in remissed elderly infected subjects, the determination of $CD4^+$ may also be good diagnostic marker to detect 'clear immunodeficiency' in aging, because of good $CD4^+$ number maintenance (700 mm^3) for good health (successful of aging) in centenarians (Sansoni et al., 1993).

The parallelism between aging and HIV may exist because, other than similar T-cell dysregulations (Ullum, Lepri, Victor, Skinhoj, Phillips & Pedersen, 1997), strong $CD4^+$ depletion associated with marked zinc-deficiency causes the appearance of 'severe' opportunistic infections, including Candida aesophagea, in HIV^+ subjects (Murray et al., 1985; Mocchegiani et al., 1995b). The pathogen agent involved is quite different between HIV and aging. Because of the lack of difference in $CD4^+$ depletion between bronchopneumonia by Candida and bacteria complication while, one hand possible different immune responses by different pathogen agents may be avoided (Belkaid et al., 1994); on the other hand, the parallelism with HIV in $CD4^+$ depletion for 'severe' infections may be further supported. Indeed supplementing zinc reduces infections relapse with low scores in $CD4^+$ and zincaemia relative risk factors in elderly infected subjects, as it occurs in HIV^+ zinc-treated subiects (Mocchegiani et al., 1995b). This suggests that zinc may induce resistence against infections (Shankar & Prasad, 1998). On the other hand resistence to respiratory and urinary infections in instituzionalized elderly subjects (Girodon et al., 1997) and in Down's syndrome (Licastro et

al., 1994) is observed during supplementing zinc. In addition, benefit of physiological zinc against gram-negative infections has been reported (Wellinghausen et al., 1996). A further evidence of zinc deficiency relevance for infections incidence comes from the AZT poor efficacy, as compared to HAART (Hazura & Kuo, 1997), in HIV condition. Indeed, HAART increases $CD4^+$ cells number and zincaemia values, which are both inversely correlated with HIV-RNA, and, consequently, there is no appearance of opportunistic infections. The disappearance of $CD4^+$ depletion and zinc deficiency as possible relative risk factors for opportunistic infections exclusively during HAART therapy (Table 2), is in line with this interpretation. The HAART effect may also occur by means of major zinc bioavailability due or to decreased acute inflammation (Chandra, 1992) or to better zinc intestinal absorption, as shown by body weight increments. Because of the absence of zinc in HAART drugs (Veccia, unpublished observation), these findings together with the effect of zinc supplementation in elderly subjects (in the present study) and in HIV^+ subjects (Mocchegiani et al., 1995b), while one hand suggest the real efficacy of HAART, on the other hand pin-point zinc as beneficial against 'severe' infections, whose effect may be independent either by HIV-virus or by other pathogen agents involved. Moreover, such an effect may be more addressed to the extracellular matrix because of the disappearance of Candida aesophagea relapse in HIV^+ zinc-treated subjects (Mocchegiani et al., 1995b). While HAART reduces infections occurrence in HIV by means of also major zinc bioavailability, this latter may be acquired by zinc supplementation in aging. Other micronutrients or vitamins deficits have been suggested as infection predictors (Chandra, 1989). Without excluding that, Vitamin A supplementation has been found deleterious on immune response in elderly people, whereas zinc prevents infections (Fortes et al., 1998)—further supporting, this latter, the relevance of zinc deficiency in infected elderly patients during the remission phase. Intestinal malabsorption (Lee, Prasad, Hydrick-Adair, Brewer, & Johnson, 1993) or inflammation (Chandra, 1992) may cause zinc loss in aging. Whatever causes, low zinc bioavailability is associated with $CD4^+$ depletion, despite zinc content into young and old human lymphocytes has been found similar (Bunker, Hinks, Lawson, & Clayton, 1984), whereas it is different for others (Prasad et al., 1993). The free quota of zinc available is more relevant because zinc into lymphocytes (Yurkow & Makhijani, 1998) is more bound with zinc-binding metallothioneins, which are increased in aging (Mocchegiani, Muzzioli, Cipriano & Giacconi, 1998) and in infections (Sobocinski, Canterbury, Mapes, & Dinterman, 1995). Since Atomic Absorption Spectrophotometry (AAS) detects zinc-bound and zinc-unbound, the measure of zinc into lymphocytes may, therefore, result controversial and, at least, also misleading. Other zinc deficiency markers, such as ecto-5 nucleotidase, are useful (Prasad et al., 1993). However, TT/AT ratio purchases peculiar role to test real zinc deficiency because TT/AT ratio detects zinc ions bioavailability due to strict inverse correlation between zincaemia status and ratio itself. Moreover TT/AT ratio > 2 ($\log_{-2}$) is always associated with more marked zinc deficiency, as compared to TT/AT ratio $= 1$ ($\log_{-2}$) in presence of normal plasma zinc values (Fabris et al., 1984). Thus the low zinc ions bioavailability is evident in aging leading to $CD4^+$ depletion and to the appearance of infections relapses. Thereby, the cross-linking between zinc deficiency and $CD4^+$ depletion in aging may be probable relative risk factor avoided by supplementing zinc.

In conclusion, present data, despite obtained in limited number of subjects, may suggest parallelism between aging and HIV because of the resemblance in $CD4^+$ depletion and zinc

deficiecy as relative risk factors for 'severe' infections relapse. Because of $CD4^+$ apoptosis involvement in both conditions (Castro et al., 1998; Algeciras, Dockrell, Lynch, & Paya, 1998), and, in turn, apoptosis may be directly (Sunderman, 1995) or indirectly (Shankar & Prasad, 1998) prevented by zinc, the resemblance may be further supported. New concepts on cause/s of 'immunosenescence' (Pawlec & Solana, 1997) may be thereby added with an emphasis on zinc.

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